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Ran-GTP-dependent Kap104p translocation through the nuclear pore complex

by

Kelly Anne Hjertaas

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Cell Biology

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Ran-GTP-dependent Kap104p translocation through the nuclear pore complex." submitted by Kelly Anne Hjertaas in partial fulfillment of the requirements for the degree of Master in Science.

I would like to dedicate this thesis to my parents for their loving support and to Russell Tedrick for his patience and support through it all.

Abstract

In *Saccharomyces cerevisiae,* Kap104p is a soluble nuclear import receptor (karyopherin) that mediates the import of two essential mRNA binding proteins through the nuclear pore complex, which penetrates the nuclear envelope and provides a conduit for macromolecular passage. Ran-GTP, the only known energy source for this process, releases importing karyopherins from their cargoes and from nucleoporins although how and where these interactions occur remains obscure. To address these issues, a mutant, kap104*355-8p, was made to attenuate the Kap104p cycle at the point where Ran-GTP is required. *In vitro* binding assays demonstrate that the mutant is deficient in Ran binding and *in vivo* approaches show an accumulation of kap104*355-8p and its cargo at the nuclear envelope. We interpret this to suggest that kap104*355-8p remains bound to specific nucleoporins from which it cannot release without Ran-GTP binding. *Ex vivo* approaches point to Nup116p as this site. These results suggest Ran-GTP is active within the nuclear pore complex and is required to release Kap104p from specific nucleoporins including Nup116p.

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First and foremost I would like to thank Dr. John Aitchison for taking me on as a graduate student and teaching me so much. I would also like to thank supervisory committee, Dr. Rick Wozniak and Dr. Rick Rachubinski, for their helpful discussions and direction with my research. And finally I would like to thank all the people who work in my lab and have helped me in ways to numerous to mention and always made it a joy to come to the lab.

Abbreviations

5-FOA	5'-fluoro-orotic acid
bp	base pairs
BSA	bovine serum albumin
cNLS	classical nuclear localization signal
D	Dalton
DAPI	diamidino-2-phenylindole dihydrochloride
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
g	gravitational force
GDP	guanosine diphosphate
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTP	guanosine triphosphate
hnRNP	heterogeneous nuclear ribonuclearprotein
IPTG	isopropyl-β-D-thiogalactoside
kap	karyopherin
mRNA	messenger ribonucleic acid
Nab	nuclear poly (A)-binding protein
NE	nuclear envelope
NES	nuclear export signal
NLS	nuclear localization signal

NPC	nuclear pore complex
Nup	nucleoporin
nt	nucleotides
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethyl sulfonyl fluoride
Poly(A)	polyadenylated RNA
Ran	ras-related nuclear protein
Ran-BP1	ran binding protein 1
RanGAP	ran GTPase activating protein
rgNLS	arginine/glycine rich NLS
RNA	ribonucleic acid
SV40lT	large tumor antigen from Simian Virus 40
TCA	trichloroacetic acid
U	units of enzyme
V	volts
v	volume
w	weight
Yrb1	yeast ran binding protein 1
Ω	ohm
ψ	pounds per square inch

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1. Introduction

1.1 The Nuclear Pore Complex

In eukaryotes, the nuclear envelope (NE) is a double membrane structure that is continuous with the endoplasmic reticulum and separates the sites of transcription and translation. This physical barrier must be overcome in a properly controlled manner to maintain cell viability. Nuclear pore complexes (NPCs) embedded in the NE form gated channels that allow the flow of molecules into and out of the nucleus. The NPC forms an aqueous channel through the NE, allowing the passive diffusion of ions, metabolites and small polypeptides (less than ~40kD; Rout and Wente, 1994; Gorlich and Mattaj, 1996), while larger polypeptides, pre-ribosomes and ribonuclear proteins are actively transported in saturable and energy-dependent pathways (Gorlich and Mattaj, 1996; Nigg, 1997).

The NPC contains eight spokes around a central channel or transporter that are attached to four rings running parallel to the plane of the NE. In general, eight filaments extend into the cytoplasm and are termed the cytoplasmic filaments, while another eight filaments extend into the nucleus and form the nuclear basket (Figure 1) (Reviewed by Rout and Wente, 1994). The overall structure is conserved among all eukaryotes; however, in yeast it appears that lumenal structures are not present (Reichelt et al., 1990; Fabre and Hurt, 1997).

A great deal of effort has been dedicated to establishing the molecular architecture of the NPC (reviewed by Rout and Wente, 1994; Ryan and Wente, 2000;

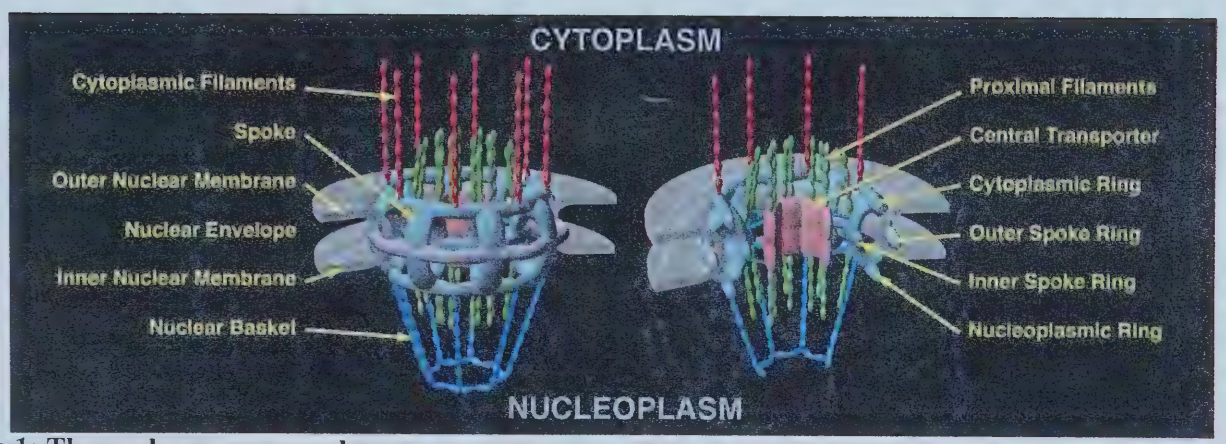


Figure 1: The nuclear pore complex.

The NPC is embedded in the nuclear envelope where the inner and outer nuclear membranes fuse. The NPC is composed of eight spokes that surround the central transporter. The spokes are attached to four coaxial rings: the cytoplasmic ring, the outer and inner spoke rings and the nucleoplasmic ring. Cytoplasmic filaments project into the cytoplasm and the nuclear basket projects into the nucleoplasm. Proximal filaments are theorized to project from the core of the NPC, but have not yet been directly imaged. The yeast NPC lacks the cytoplasmic and nucleoplasmic rings present in vertebrate NPCs, at least partially accounting for the difference in mass between yeast and vertebrate NPCs. The figure is from Rout and Aitchison, 2001.

Wente, 2000; Rout and Aitchison, 2001). Recently, we took a comprehensive proteomics approach toward this goal (Rout et al., 2000). Incorporating these data and those of many laboratories, the following picture emerged: the NPC of *Saccharomyces cerevisiae* consists of ~30 proteins, termed nucleoporins or nups, and has a molecular mass of ~44 Mega Daltons (Rout et al., 2000). Most of the nucleoporins are present in multiple copies and are located symmetrically in the NPC. The few asymmetric nucleoporins have been suggested to be components of the cytoplasmic filaments or nuclear basket, contributing to the morphological differences between the two faces of the NPC.

Nucleoporins can be divided into two categories, those that contain a degenerate phenalanine-glycine (FG) repeat and those that do not. In general, the 12 FG containing nucleoporins in yeast are more perpherally positioned and are believed to play an active role in nucleocytoplasmic transport by providing docking sites for specific soluble transporters. By comparison, the non-FG-containing nucleoporins generally provide a scaffold upon which the more peripherally desposed FG-nups are arranged. Nevertheless, these generalities do not hold true in all cases. For example, Nup82, a non-FG nucleoporin appears to form part of the cytoplasmic filaments and is required for mRNA transport (Hurwitz and Blobel, 1995).

Interestingly, some nucleoporins are more dynamic within the NPC than originally thought. For instance, in yeast, Nup2p was originally classified as a typical nucleoporin for several reasons. Nup2p contains FG repeats, localizes to the NPC by

immunofluorescence microscopy and more specifically to the nuclear basket by immunoelectron microscopy (Loeb et al., 1993; Booth et al., 1999; Hoods et al., 2000; Dilworth et al., 2001). However, sub-cellular fractionation has shown Nup2p to be only weakly associated with NPCs (Aitchison et al., 1996; Rout et al., 2000). Recent heterokaryon experiments have shown that this weak association is due to Nup2p being mobile within the NPC and associating with both distal faces of the NPC (Dilworth et al., 2001). Nakielny et al. (1999) found similar results with mammalian Nup153p, suggesting individual nucleoporins in yeast and vertebrates may transiently associate with the NPC.

1.2 Karyopherins

Although extensively studied, exactly how the NPC governs the movement of macromolecules between the nucleus and the cytoplasm is unclear. However, it is clear that most macromolecules are specifically transported across the NPC by a group of proteins collectively called karyopherins (derived from the Greek words karnon for 'nucleus' and pher (ein) meaning 'to bring to' or 'to carry from'; Radu et al. 1995). Specific karyopherins have also been termed, importins/exportins/transportins, but all members of the karyopherin family recognize macromolecules containing the correct "signal" and mediate their transport.

Proteins destined to cross the NPC contain a nuclear localization signal (NLS) or a nuclear export signal (NES) that is recognized by the specific karyopherins. The first NLS to be identified was a short sequence of basic amino acids within Simian

Virus (SV) 40 large T antigen (SV40lT; Kalderon et al., 1984a). Further studies revealed that a minimum sequence of PKKKRKV was capable of mediating the import of non-nuclear proteins and that any mutations in this sequence abolished protein import (Kalderon et al., 1984b; Lanford and Butel, 1984). Since this NLS was discovered, other proteins containing stretches of basic amino acids acting as NLSs have been identified, and collectively these nuclear signals are termed classical nuclear localization signals (cNLSs) (Dingwall and Laskey, 1991). A heterodimer, consisting of Kapα and Kapβ, was identified and found to be required for the import of the cNLS (also known as importin α/β or Kap 60/95 in yeast) (Adam and Gerace, 1991; Adam and Adam, 1994; Gorlich et al., 1994; Gorlich et al., 1995a; Chi et al., 1995; Imamoto et al., 1995; Radu et al., 1995). In the cytoplasm, Kapα binds the cNLS-containing protein (Gorlich et al., 1994) and acts as an adaptor for Kapβ. Kapβ then mediates complex docking to, and movement through, the NPC (Gorlich et al., 1995b; Moroianu et al., 1995).

In vertebrate systems, several α subunits have been found that can individually associate with one β subunit (Moroianu et al., 1995; Radu et al., 1995; Kohler et al., 1999). In contrast, only one (Kap60/Srp1p) α subunit is present in yeast. Kap60/Srp1p was originally discovered as a suppressor of temperature sensitive RNA polymerase I mutants (*SRP1*; Yano et al., 1992). When a Kap60/Srp1p protein A chimera was immunoisolated, its association with a 95 kD protein was discovered. The 95 kD protein was shown to have homology to rat Kapβ, was capable of directing the docking of cNLS-containing proteins in

mammalian cells (Enenkel et al., 1995) and was thus designated Kap95p. It was shown that Kap60/Srp1p provided the cNLS-binding site (Adam and Gerace, 1991; Gorlich et al., 1994) while, Kap95p, in turn, binds to Kap60p and enhances the affinity of Kap60p for the cNLS (Rexach and Blobel, 1995; Enenkel et al., 1996).

While the import of cNLS-containing proteins by Kapα/β is well studied and, consequently, is from where most of our understanding of nuclear import comes, numerous proteins that must cross the NPC do not all contain a cNLS and, therefore, are not substrates for Kapα and Kapβ. Alternative pathways across the NE that allow import and export of many of those substrates have since been discovered (Table 1.1; Figure 2). In yeast, 14 different members of the Karyopherin β family have been identified based on their sequence similarity to Kap95p (Mattaj and Englmeier, 1998; Pemberton et al., 1998; Wozniak et al., 1998). The first member of this family to be characterized was Kap104p (also known as Kapβ2 or Transportin (Trn1) in humans) (Aitchison et al., 1996; Pollard et al., 1996). Kap104p was shown to mediate the import of two essential heterogeneous nuclear ribonucleur proteins (hnRNPs) termed Nab2p and Nab4/Hrp1p (Aitchison et al., 1996; Lee and Aitchison, 1999). Kap104p interacts directly with Nab2p or Nab4/Hrp1p by an arginine/glycine rich region (Lee and Aitchison, 1999). This region was found to be sufficient to cause nuclear targeting of non-nuclear proteins, was distinct from the cNLS import pathway and was termed the rgNLS (Lee and Aitchison, 1999).

SGD name	Other names	Substrates

Import		
KAP95	Importin β1/Karyopherin β1	Importin α/Kayropherin α – cNLS
KAP104	Transportin/Importin β2/Karyopherin β2	mRNA binding proteins – Nab2p, Nab4/Hrp1p
KAP121	RanBP5/PSE1/ Karyopherin β3	Ribosomal proteins
KAP123	Yrb4/Karyopherin β4	Ribosomal proteins
SXM1	Kap108	Lhp1p, ribosomal proteins
KAP120		Ribosomal proteins
MTR10	Kap111	Hrp1p, Npl3p
NMD5	Kap119	Hog1p, TFIIs
PDR6	Kap122	TFIIa
KAP114		TBP, Histones – H2A, H2B
Export		
CSE1	CAS/Kap109	Import α/Karyopherin α
CRM1	Exportin 1/Xpo1/Kap124	Leucine-rich NESs
LOS1	Exportin-t/Kap127	tRNA
Bi-directional Transport		
MSN5	Kap142/Exportin 5	Import: Rpa complex Export: Pho4p, Mig1p, Far1p

Table 1.1 Members of the Karyopherin β family and their known substrates. (Adapted from Chook and Blobel, 2001)

Karyopherins mediate the transport of macromolecules by overcoming the exclusion barrier of the NPC. Exactly how this is done is unknown, although it is presumably through karyopherin-nucleoporin interactions. As mentioned above, several nucleoporins contain FG repeats. These FG nucleoporins are believed to have

a direct role in transport by acting as binding sites for karyopherins traversing the NPC. In fact, Bayliss et al. (2000) determined the crystal structure of Kapβ interacting with the FG repeats of Nsp1p and showed that deletion of the FG binding region of Kapβ impaired import. The models for how the NPC gated function works invoke these karyopherin/nucleoporin interactions to explain potential mechanisms for karyopherin movement through the NPC.

Rout et al. (2000) suggest that the NPC acts as an entropic barrier to diffusion, which can be specifically overcome by karyopherins. In this model, the movement of molecules into the narrow aperture of the pore is entropically, and therefore energetically, unfavorable because so doing constricts the molecule's degrees of freedom. However, if molecules have a binding affinity for components of the channel, this entropic loss can be compensated by the potential energy associated with binding. In this way, selective binding creates a virtual gate. The binding sites for karyopherins are generally found on filamentous FG-containing nucleoporins, which, as mentioned above, are positioned at entry/exit sites on both sides of the NPC and surround the central channel. Furthermore, in this model, the Brownian motion of the filamentous, disordered FG-nups provides an additional entropy component that must be overcome by macromolecules entering the pore. Ribbeck and Gorlich (2001), on the other hand, suggest that FG nucleoporins interact across the central transporter forming a meshwork. This meshwork is thought to be hydrophobic, thereby creating a permeability barrier. Smaller macromolecules are predicted to be able to fit between the interacting FG nups, while larger macromolecules would be

excluded or repelled by the hydrophobicity (Ribbeck and Gorlich, 2001). The karyopherin/nucleoporin interactions, however, are suggested to be hydrophobic-based and allow karyopherins and their substrates to enter the meshwork. The karyopherins and their substrates would "melt into" the meshwork, allowing the NPC to exclude macromolecules even as one is passing through it. Again, multiple binding and release steps are proposed to occur as the karyopherin and its substrate move through the NPC. In both models, the ability of karyopherins to interact with nucleoporins, in particular FG nucleoporins, are required to overcome the exclusion barrier created by the NPC, while they do not require an actual mechanistic gate to be opened or closed.

Karyopherins have also been shown to have different affinities for different nucleoporins (Reviewed by Ryan and Wente, 2000), and it has been suggested that karyopherins have the highest affinity for nucleoporins at the terminal steps of transport (Rout et al., 2000). In support of this, Ben-Efraim and Gerace (2001) showed that Kapβ interacts with Nup358p, Nup62p and Nup153p, but that it has the highest affinity for Nup153p, a nucleoporin in the nucleoplasmic regions of the NPC. Similar interactions have been shown in yeast, with Kap60p and Kap95p interacting with Nup60p and Nup1p, both nucleoporins at the nuclear face of the NPC (Marelli et al., 1998; Dilworth et al., 2001). Additionally, the export karyopherin Crm1p has been shown to interact with Nup42p and Nup159p, which are present only on the cytoplasmic face of the NPC (Jensen et al., 2000; Allen et al., 2001). Together, these

findings suggest the existence of high-affinity binding sites that may promote karyopherin movement through the NPC and contribute directionality to transport.

However, not all karyopherins appear to interact with highest affinity to asymmetric nucleoporins. Kap121p interacts specifically with the symmetrically disposed nucleoporin Nup53p (Marelli et al, 1998). In addition, Kap104p interacts with several symmetrically positioned nucleoporins, but specific interactions with nuclear biased nucleoporins (Aitchison et al., 1996; Allen et al., 2001) have not been reported. The ability to interact with nucleoporins presumably allows these karyopherins to overcome the exclusion barrier of the NPC; however, the lack of an obvious interaction with a nucleoporin on the nuclear face of the NPC makes it unclear how these karyopherins move from the interior of the NPC to the nucleoplasm.

1.3 The Role of Ran

The only known energy source for nuclear transport is a small GTPase termed Ran (ras-related nuclear protein) or Gsp1p (GTP-binding suppressor of prp20) (Belhumeur et al., 1993; Melchior et al., 1993; Moore and Blobel, 1993), which cycles between a GTP bound form and a GDP bound form. A nuclear-restricted GTP exchange factor termed Prp20p (pre-RNA processing) in yeast, and RCC1 (regulator of chromosome condensation) in vertebrates, promotes the exchange of GDP for GTP (Seki et al., 1996). In the cytoplasm, Ran binding protein 1 (RanBP1) (also known as yeast ran binding protein 1 (Yrb1p) in *S. cerevisiae*) (Bischoff et al., 1995; Hayashi et

al., 1995; Schlenstedt et al., 1997), works with a cytoplamic Ran GTPase-activating protein called RanGAP1 in vertebrates (Rna1p in yeast) to stimulate Ran-GTPase activity (Bischoff et al., 1995a; Traglia et al., 1996). The cellular localization of these Ran regulators creates a Ran gradient, with high concentrations of Ran-GTP in the nucleus and high concentrations of Ran-GDP in the cytoplasm (Gorlich et al., 1996a; 1996b). The maintenance of this gradient defines the cellular compartments and helps provide directionality to transport (Gorlich and Mattaj, 1996; Moore, 1998; Gorlich and Kutay, 1999). For instance, the import complex Kapα/β/cNLS is stable in the presence of Ran-GDP (Figure 2) (Rexach and Blobel, 1995). However, once imported into the nucleus, the presence of Ran-GTP triggers the dissociation of the complex (Rexach and Blobel, 1995). Nuclear export works in an analogous fashion, with complex formation occurring in the nucleus in the presence of Ran-GTP, and, upon hydrolysis of GTP on Ran in the cytoplasm, dissociation is triggered (Figure 2) (Fornerod et al., 1997). Another factor termed Ntf2/p10 is also required for nuclear import (Nehrbass and Blobel, 1996). It is proposed that Ntf2/p10 functions by recycling Ran-GDP to the nucleus (Ribbeck et al., 1998; Quimby et al., 2000a; 2000b).

In addition to stimulating substrate release in the proper cellular compartment, Ran-GTP also affects karyopherin/nucleoporin interactions (Rexach and Blobel, 1995; Marelli et al., 1998; Nakielny and Dreyfuss, 1999; Seedorf et al., 1999; Allen et al., 2001). As a result, it has been suggested that Ran acts within the NPC, helping to promote the release of transport complexes from nucleoporins as they move through

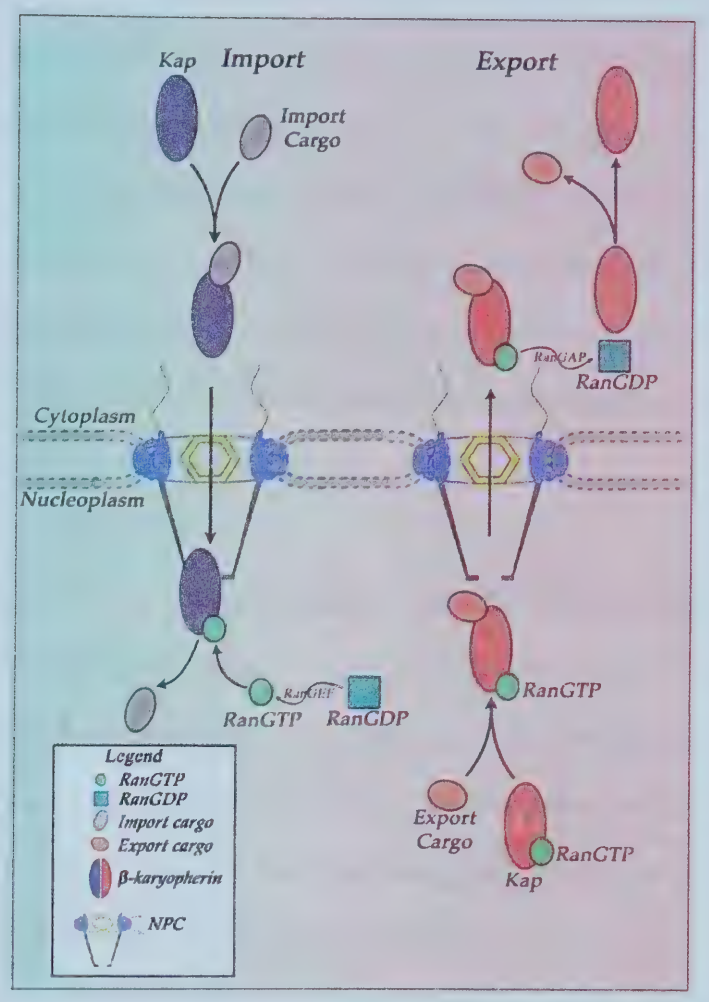


Figure 2: Karyopherin-mediated nuclear transport.

Import: A specific karyopherin forms a complex with its cargo and mediates complex docking to the NPC. Interactions between the karyopherin and FG-nups facilitate translocation through the NPC. Upon entry to the nucleus, high levels of Ran-GTP stimulate dissociation of the import complex. RanGEF (Prp20p in yeast) maintains the high levels of Ran-GTP within the nucleus. The karyopherin is then recycled back to the cytoplasm for further rounds of import. Export: The export complex forms in the presence of Ran-GTP and the karyopherin mediates translocation through the NPC in a fashion similar to that of import. Within the cytoplasm, the Kap/cargo/Ran-GTP complex dissociates when Ran-GTP is hydrolyzed by RanGAP (Rna1p in yeast). The figure is from Marelli et al., 2001.

the NPC (Rexach and Blobel, 1995; Seedorf et al., 1999; Allen et al., 2001). In support of this theory, immunoelectron microscopy experiments have revealed Ran associated with the NPC (Melchoir et al., 1995; Wu et al., 1995; Yanseen and Blobel, 1999). However, it is not clear if Ran is active within the NPC *in vivo* or if its detection is a result of its transport by Ntf2/p10 in an inactive Ran-GDP form. If Ran-GTP is active within the NPC, it poses many questions. For example, if Ran-GTP is active within the NPC how can it release karyopherins from nucleoporins without releasing cargoes before reaching the nucleoplasm?

Although Ran is the only known energy source for nuclear transport, recent experiments have shown that a single round of transport *in vitro* does not require Ran or GTP hydrolysis (Schwoebel at al., 1998; Englmeier et al, 1999; Ribbeck et al., 1999). However, it is not clear if transport has the same requirements *in vivo*. In the above experiments the components were added to a permeabilised cell system, and import or export monitored. It is possible the components were added in excess, and, as a result, their own concentration gradient provided the impetus for transport. However, these results have been interpreted to suggest that energy is not required for the translocation of transport complexes; rather, Ran and GTP hydrolysis may be required for the recycling of karyopherins.

Studies of Kapβ/Kapα/cNLS import suggested Ran-GTP is required at the nuclear face of the NPC (Hoods et al., 2000; Solsbacher et al., 2000). However, studies of other karyopherins conflict with this. Three karyopherins in yeast have

been shown to require Ran-GTP and additional factors for efficient cargo release, presumably at sites within the nuclear interior. Kap114p imports a TATA-binding protein (TBP), and, in addition to Ran-GTP, the presence of double-stranded TATA-containing DNA is required to stimulate complex dissociation (Pemberton et al., 1999). Mtr10p imports the hnRNP Npl3p, which requires RNA and Ran-GTP to be present for efficient cargo release (Senger et al., 1998). Kap104p has also been shown to require Ran-GTP and mRNA for efficient release of its cargo (Lee and Aitchison, 1999). Therefore, precisely where Ran-GTP is required in these import pathways remains unclear. It is possible Ran-GTP acts at the NPC to help these import complexes release nucleoporins and move towards the nuclear interior and this activity is not sufficient to release cargo. Once in the nucleus, Ran-GTP may work cooperatively with the other elements to cause cargo release. However, it is equally possible that Ran-GTP is not required at the NPC, but rather, it may only be required within the nucleus where cargo is released.

1.4 Thesis Objectives

The conservation of nuclear transport among species allows us to extrapolate information learned in one species and apply that information to other species. In this thesis, the crystal structure of Kapβ2/Trn1 (Chook and Blobel, 1999) was used to introduce changes into Kap104p, creating a mutant that is deficient in Ran-GTP binding. As mentioned above, previous studies have shown that Ran-GTP is required in addition to mRNA for efficient dissociation of the Kap104p import complex (Lee and Aitchison, 1999). However, it remains unclear where Ran-GTP is required for

the completion of Kap104p-mediated import. Ran-GTP may be required at the NPC to allow the complex to release from the nucleoporins, or Ran-GTP may be required at the site of mRNA transcription. There is also the possibility that Ran-GTP is required only for the export of Kap104p after it has imported its cargo. *In vitro* and *in vivo* approaches using the developed mutant provide insight into the role of Ran-GTP in Kap104p-mediated import.

2 Materials and Methods

2.1 DNA Amplification and Isolation

DNA fragments of interest were amplified by the Polymerase Chain Reaction (PCR). All DNA amplifications were done using Expand HF Polymerase (Roche), following the manufacturer's specifications. Primers were customized for each application (See Table 2.1). As a template, 50-100 ng of yeast genomic DNA library (Promega) or 100 ng of a purified plasmid containing the required DNA fragment were used. PCR products were extracted with 1 volume choloroform (BDH Inc.), subjected to centrifugation for 3 minutes at 11,250 x *g* at 4°C. The top aqueous layers were moved to a clean tube and 2.5 volumes of 100% ethanol (Commercial Alcohols Inc., Brampton, Canada) and 0.1 volume 3 M sodium acetate, pH 5.5 were added to precipitate the PCR products. Samples were incubated at –20°C for 30 minutes and subjected to centrifugation for 30 minutes at 11,250 x *g* at 4°C. Precipitated DNA was washed with 70% ethanol, dried by vacuum centrifugation and resuspended in water.

Oligonucleotide Name	Sequence	Comments
Ybr-325		Oligonucleotide was used to create kap104*355-8-pGEM-T and kap104*355-8-pRS315. Aitchison et al., 1996
#752	5' gga gaa ttc gag ctc aaa tgg cat cga cat gga agc cc 3'	Oligonucleotide was used to create kap104*355-8-pGEM-T, kap104*355-8-pYES2 and Kap104-pYES2. (Aitchison et al., 1996)
Kap104M#1 3'	5' gag gat aaa gat TCg CGc TCc GGg ccc GGC gcC ccc cgt att gtg aaa aa 3'	Oligonucleotide is reverse complement of this sequence. This oligonucleotide was used to introduce site-specific changes to *KAP104*355-8* in pGEM-T (changes indicated by capitals).

Kap104WT#1 5'	5' c aag ccc GGC gcC ccc cgt att gtg aaa aag aaa gag gca 3'	The KasI site here overlaps with the KasI site in the Kap104M#1 3' (changes indicated by capitals) and was used to make kap104*355-8-pGEM-T.
Kap104 (b/s)3'	5' ttc tta caa cag ttc acc agc taa gga tcc cgc gag ctc ccg 3'	Oligonucleotide is reverse complement of this sequence. This oligonucleotide was used to subclone *KAP104*355-8* into pGEM-T, pGEX4T-1 and pYES2 and to subclone *KAP104* into the pYES2 vector.
Kap104(bam)5'	5' cgt gga tcc ggg atg gca tcg aca tgg aag ccc 3'	This oligonucleotide was used to subclone *KAP104*355-8* into the pGEX4T-1 vector.
Kap104#1 5'	5' tac ggg atc cat cga atg gca tcg aca tgg aag ccc 3'	This oligonucleotide was used to subclone *KAP104*355-8* into the pGBKT7 and *KAP104* into pGBT9.
Kap104#1 3'	5' ttc tta caa cag ttc acc agc taa ttc ccg ggt ggg atc cat 3'	Oligonucleotide is the reverse complement of this sequence. It was used to subclone *KAP104*355-8* into the pGBKT7 and *KAP104* into pGBT9.
Kap104 5'	5' gcg caa gag atc tac acc ttt ttg atg 3'	This oligonucleotide was used to create kap104*355-8-prA- and Kap104-prA-pYES2 vectors.
ProtA 3'	5'cat agt tta gcg gcc gct caa gga tcg tct tta agg ctt tg 3'	Oligonucleotide is the reverse complement of this sequence. It was used to create kap104*355-8-prA- and Kap104-prA-pYES2 vectors.
Sma/Eco Nup-42 ATG	5' cc cgg gaa ttc atg tca gct ttc ggt aac 3'	This oligonucleotide was used to create the Nup42-pGADT7 vector.
Sma/Eco Nup-42 TAG	5' ccc ggg gaa ttc cta tgc aac caa tgc agg 3'	Oligonucleotide is the reverse complement of this sequence. This oligonucleotide was used to create the Nup42-pGADT7 vector.

Table 2.1: Oligonucleotidenucleotide sequences and uses

2.2 Sub-cloning of DNA Fragments

Generally, DNA was digested with commercial restriction enzymes at 37°C as per the manufacturer's instructions. Digested fragments were separated on a 1% GTG agarose gel (FMC, Rockland, ME) in 1% TBE (Tris-Borate EDTA). The desired band was cut out and the DNA isolated using a Gel Extraction Kit (Qiagen). Isolated

fragments were ligated in 10 µl total volume reactions (200ng total DNA per 10 µl reaction) at 16°C overnight using 1 U T4 DNA ligase (Boehringer Mannheim) with 1 µl 10X T4 DNA ligase buffer (Boehringer Mannheim). Approximately 4:1 insert:vector molar ratio was used in each reaction.

2.3 Bacterial Transformation and Plasmid Extraction

Heat shock competent cells (100 µl) were mixed with either 1 µl of plasmid DNA or 3 µl of the ligation reaction mix (See Table 2.2). Cells were incubated for 30 minutes on ice and were then heat shocked for 3 minutes at 37°C. Cells were then put back on ice for 2 minutes and added to 900 µl of SOB medium (Manniatis et al., 1992) for recovery at 37°C for 45 minutes with agitation, prior to plating on the desired media.

Contruct	Gene	Oligonucleotides Used	Restriction Enzymes	Gene Source
kap104*355-8-pGEM-T (step 1)	*KAP104* nt #–325 or #1 to #1100	Ybr325 or 752 and 104M#1 3'		Yeast genomic DNA (Promega)
kap104*355-8-pGEM-T (step 2)	*KAP104* Nt #1071 to #2757	104wt 5' and 104(b/s) 3'		Yeast genomic DNA (Promega)
kap104*355-8-pGEM-T (final)	*KAP104* Nt #-325 or #1 To #2757		KasI/SacI	Step 1 and Step 2
kap104*355-8-pRS315	*KAP104*355-8*	Ybr325 and 104(b/s) 3'	SacI	kap104*355-8-pGEM-T (final)
kap104*355-8-pGEX4T-1	*KAP104*355-8*	104(bam) 5' and 104(b/s) 3'	BamHI	kap104*355-8-pGEM-T (final)
kap104*355-8-pYES2	*KAP104*355-8*	752 and 104(b/s) 3'	SacI	kap104*355-8-pGEM-T (final)
kap104*355-8-prA-pYES2	*KAP104-prA*	Kap104 5' and ProtA 3'	BglII and NotI	Yeast genomic DNA
kap104*355-8-	*KAP104*355-8*	104#1 5' and	BamHI	kap104*355-8-

pGBKT7		104#1 3'		pGEM-T (final)
Kap104-pYES2	*KAP104*	752 and 104(b/s) 3'	SacI	Kap104-pRS317
Kap104-prA-pYES2	*KAP104-prA*	Kap104 5' and ProtA 3'	BglII and NotI	Yeast genomic DNA
Kap104-pGBT9	*KAP104*	104#1 5' and 104#1 3'	BamHI	Kap104-pRS317 (Aitchison et al., 1996)
Nup42-pGADT7	*NUP42*	Sma/Eco Nup42 ATG and Sma/Eco Nup42 TAG	SmaI and EcoRI	Yeast genomic DNA (Promega)
Nab2-pGEX2T-K				Lee and Aitchison, 1999
Kap104-pGEX2T-K				Lee and Aitchison, 1999
Ran-pGEX				Floer and Blobel, 1996
Kap104-pRS317				Aitchison et al., 1999

Table 2.2: Plasmids and constructs

Single colonies were used to inoculate 3 ml overnight cultures. Cultures were collected, the media aspirated, and the cell pellets resuspended in 100 µl TE + RNAase (50 mM Tris-HCL pH 7.5, 10 mM EDTA, 100 ug/ml RNAase A). Samples were incubated on ice for 5 minutes before 200 µl of 0.2 M NaOH/1% SDS were added and samples inverted several times to mix. After incubation on for 5 minutes 150 µl of 3 M Potassium (BDH Inc.) 5 M Acetate (Fischer Scientific) were added and samples mixed by inversion until a white precipitate was visible. Samples were incubated on ice for 10 minutes followed by centrifugation for 10 minutes at 4°C at 11,250 x *g*. The supernatants were moved to a clean tube and an equal volume of phenol:chloroform (Life Technologies) (BDH Inc.) mixture (50:50) added. Samples were mixed using a vortex for 5 seconds followed by centrifugation at 4°C, 11,250 x *g* for 3 minutes. The aqueous top layer was moved to a clean tube, an equal volume

of chloroform (BDH Inc.) added and samples mixed using a vortex and subjected to centrifugation as above. The aqueous top layer was then moved to a clean tube, 1 ml of 100% ethanol added, mixed by inversion and incubated at –20°C for 30 minutes. Samples were subjected to centrifugation at 4°C at 11,250 x *g* for 30 minutes, washed with 70% ethanol and the pellets dried by vacuum centrifugation. The pellets were then resuspended in 30 µl of water.

2.4 Construction of kap104*355-8pGEM-T

The kap104*355-8p mutant was made by site-directed mutagenesis and constructed with or without the endogenous promoter. Both forms were generated in 3 steps. In the first step, PCR was used to amplify the *KAP104* sequence from nt +1 to nt 1100 or from nt –325 to nt 1100. The 5' primer contained a SacI restriction site (Ybr-325 or #752; see Table 2.1). The 3' primer contained the specific nucleotide changes desired was well as an introduced KasI restriction site (Kap104M#1 3', see Table 2.1). After amplification the fragment was ligated into the pGEM-T vector (Promega, Madison, WI) (see Table 2.2). The second step involved the PCR amplification of *KAP104* from nt 1071 to nt 2757, generating a fragment with the introduced KasI restriction site at the 5' end (Kap104WT#1 5', see Table 2.1) and a SacI restriction site at the 3' end (Kap104(b/s) 3', see Table 2.1). This fragment was also ligated into the pGEM-T vector (see Table 2.2). The third step involved joining the two halves of *KAP104*355-8* into the same pGEM-T vector. The second fragment was ligated into the KasI and SalI restriction sites of the pGEM-T vector containing the first fragment of *KAP104*355-8* (see Table 2.2). Dideoxynucleotide

sequencing was used to insure the correct nucleotide changes had been incorporated and that no other changes had been introduced.

2.5 Construction of kap104*355-8-pRS315, kap104*355-8-pGEX4T-1, kap104*355-8-pYES2, kap104*355-8-pGBKT7, Kap104-pGBT9 and KAP104-pYES2

Cloning the *KAP104*355-8* sequence into all other plasmids was done using the kap104*355-8-pGEM-T made above as the template or source (see Table 2.2). kap104*355-8-pRS315 was constructed by ligating the *KAP104*355-8* gene plus the 325 nucleotides upstream of the start codon into the SacI restriction site of pRS315 CEN/LEU2 (Sikorski and Hieter, 1986). kap104*355-8-pGEX4T-1 was constructed by amplifying the *KAP104*355-8* gene from pGEM-T by PCR (Kap104(bam) 5' and Kap104(b/s) 3'; see Table 2.1) and ligating it into the BamHI restriction site of pGEX4T-1 (Pharmacia Biotech Inc.) (see Table 2.2). kap104*355-8-pYES2 was constructed by amplifying the *KAP104*355-8* gene from pGEM-T by PCR (#752 and Kap104(b/s) 3'; see Table 2.1) and ligating it into the SacI restriction sites of pYES2 (Invitrogen) (see Table 2.2). kap104*355-8-pGBKT7 was constructed by amplifying the *KAP104*355-8* gene from pGEM-T by PCR (Kap104#1 5' and Kap104#1 3'; see Table 2.1) and ligating it into the BamHI restriction site of pGBKT-7 (Clonetech Laboratories Inc.) (see Table 2.2). The wild type *KAP104* gene was amplified from a *KAP104*-containing pRS317 vector (Aitchison et al., 1996) by PCR amplification and ligated into the SacI restriction site of pYES2 (#752 and Kap104(b/s) 3'; see Table 2.1 and 2.2) or the BamHI restriction site of pGBT9 (Kap104#1 5' and Kap104#1 3',

see Table 2.1 and 2.2). The correct orientation of all ligation products was determined by restriction digestion.

2.6 Construction of kap104*355-8-prA-pYES2 and KAP104-prA-pYES2

Genomic DNA was isolated from a Kap104-prA strain where the IgG binding domains of *Staphylococcus aureus* protein A (prA) were genomically integrated in-frame with the COOH terminus of Kap104 (Aitchison et al., 1996). One ng of genomic DNA was used as a template to amplify a fragment containing a BglII site (Kap104 5'; see Table 2.1), the prA sequence and a NotI site (ProtA 3'; see Table 2.1). The amplified product was then directionally ligated into the BglII and NotI sites of either kap104*355-8-pYES2 or Kap104-pYES2 (see Table 2.2).

2.7 Construction of Nup42-pGADT7

PCR amplification was used to amplify *NUP42* from genomic DNA, creating a fragment containing SmaI and EcoRI restriction sites at each end (see Table 2.1). The gene was then subcloned into the EcoRI restriction sites of pGADT7 (see Table 2.2) and the correct orientation determined by restriction digestion before use.

2.8 Yeast Strains and Media

All strains used are listed in Table 2.3. Unless otherwise noted all strains were grown at 30°C in YPD (1% yeast extract, 2% bactopeptone and 2% glucose) (Sherman et al., 1986) or in synthetic media (SM) with the necessary amino acids or

nucleotides and 2% glucose. 5-Fluoro-orotic acid (5-FOA) was prepared in SM as described in Ausubel et al. (1992).

Strain name	Genotype	Reference
W303α	*Matα, ade2-1, ura3-1, his3-11, trp1-1, leu2-3, 112, can1-100*	
DF5	*Mata/Matα, lys2-801/lys2-801, leu2-3, 112/ leu2-3, 112, ura3-1/, ura3-1, his3-11/ his3-11, trp1-1/ trp1-1*	
AH109	*Mat a, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, ura3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ;*	Clonetech Laboratories Inc
kap104-16	*Mata/Matα, lys2-801/lys2-801, leu2-3, 112/ leu2-3, 112, ura3-1/, ura3-1, his3-11/ his3-11, trp1-1/ trp1-1, KAP104::ura3::HIS3 + pKAP104-URA*	Aitchison et al., 1996
kap104Δ	*Mata/Matα, lys2-801/lys2-801, leu2-3, 112/ leu2-3, 112, ura3-1/, ura3-1, his3-11/ his3-11, trp1-1/ trp1-1, KAP104::ura3::HIS3*	Aitchison et al., 1996
Nup116-prA	*Mata/Matα, lys2-801/lys2-801, leu2-3, 112/ leu2-3, 112, ura3-1/, ura3-1, his3-11/ his3-11, trp1-1/ trp1-1, NUP116-prA-HIS5*	Rout et al., 2000
Kap104-prA	*Mata/Matα, lys2-801/lys2-801, leu2-3, 112/ leu2-3, 112, ura3-1/, ura3-1, his3-11/ his3-11, trp1-1/ trp1-1, NUP116-prA-HIS5*	Aitchison et al., 1996

Tabel 2.3: Yeast Strains

2.9 Yeast Transformation

The desired yeast strain was grown in a 3 mL culture overnight at 30°C with shaking. The culture was added to 50 ml of fresh media and grown for 5 hours at 30°C with shaking. Cells were pelleted by centrifugation at 2,200 x *g* for 5 minutes. Media were discarded and the cells washed twice with 1 ml of sterile water (subjected to centrifugation at 2,000 x *g* for 1 minute each time). Cells were then washed twice, as above, with ice-cold 1 M sorbitol, and finally resuspended in a minimum volume

of ice-cold 1 M sorbitol. One µl of DNA was added to 45 µl of cell suspension and loaded into the ice-cold electroporation cuvette (0.2 cm gap width, BIORAD) and electroporated (BIORAD Gene Pulser 1.5 kV, 200 Ω, 25 uFD). Cells were immediately put into 150 µl of ice-cold 1 M sorbitol and plated onto the required selective medium and grown at 30°C.

2.10 Growth Curve

W303 haploid cells containing Kap104-pYES2 or kap104*355-8-pYES2 were grown overnight in SM containing 2% glucose. Cells were then washed with water several times to remove glucose and placed in SM containing 2% galactose. At the indicated time points 6 ml were collected and 1 ml was used to determine absorbance (OD_{600}) using a spectrophotometer (Ultraspec 3000, Pharmacia Biotech). The remaining 5 ml were used to examine Kap104p and kap104*355-8p expression. The 5 ml samples were pelleted by centrifugation, 2,200 x *g* for 5 min. The supernatants were discarded and the pellets were resuspended in 240 µl 1.85 M NaOH/7.4% β-mercaptoethanol. After incubation on ice for 5 minutes, 240 µl of 50% trichloroacetic acid (TCA) were added and samples were incubated for another 5 minutes on ice. The proteins were pelleted by 11,250 x *g* centrifugation for 10 minutes at 4°C. The pellets were washed with water and resuspended in 50 µl each of Magic A (13% SDS, 1 M unbuffered Tris) and Magic B (30% glycerol, 0.25% bromophenol blue). Samples were heated for 15 minutes at 65°C and peptides separated by SDS-PAGE and analyzed by immunoblotting with Kap104p specific antibodies and enhanced chemiluminescence (ECL).

2.11 *In Vitro* Assays

2.11.1 Isolation of Recombinant Proteins

E. coli BL21 DE3 LysS (Novagen) cells transformed with either KAP104-pGEX2T-K (Lee and Aitchison, 1999), kap104*355-8-pGEX4T-1, Nab2-pGEX2T-K (Lee and Aitchison, 1999) or Ran-pGEX (Floer and Blobel, 1996) were grown over-night in 100 ml of Luria-Bertani medium + 0.1 mg/ml Ampicillin (LB+AMP) (Manniatis et al., 1992) at 37°C with shaking. Two hundred ml of fresh media were added and after 30 minutes at 37°C with shaking, IPTG (isopropyl-β-D-thiogalactoside) (Life Technologies) was added to a final concentration of 2 mM. Cells were incubated for an additional 5 hours at 37°C before collection by centrifugation at 3,000 x *g* for 10 minutes at 4°C. The supernatants were discarded and cells were washed twice with water. The pellets were frozen with dry ice/ethanol or stored overnight at –70°C. Frozen cells were resuspended in 30 ml of 1X Transport Buffer (20 mM HEPES-KOH pH 7.4, 110 mM KOAc, 2 mM $MgCl_2$, 1 μM $CaCl_2$, 1 μM $ZnCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20) and a 0.001 volume of solution P (0.4 mg/ml Pepstatin A, 18 mg/ml phenylmethysufonyl fluoride) (Aitchison et al., 1996). Samples were immediately lysed by passage through an ice-cold French pressure cell (2000ψ Sim-Aminco Spectromic Instruments) three times. Lysates were cleared by centrifugation at 2,500 x *g* for 15 minutes at 4°C. Supernatants were collected and subjected to centrifugation again at 17,000 x *g* for 20 minutes at 4°C. Supernatants were collected and stored at –70°C until needed. Ran-

GST lysates were made in the same manner but Binding Buffer (2 mM MgOAc, 150 mM KAc and 20 mM HEPES pH 6.8) was used instead of Transport Buffer.

2.11.2 Ran Loading

Two ml of lysates containing Ran-GST were incubated with 75 µl of glutathione-resin (GT; Pharmacia) for 1 hour at 4°C with rotation. The resin, with Ran-GST bound, was washed 3 times with Binding Buffer, once with Binding Buffer-500 mM NaCl and 3 more times with Binding Buffer. The Ran-resin was resuspended in 180 µl of Binding Buffer and 60 U of Thrombin (Sigma) was added to cleave off GST and release Ran. Samples were then incubated for 30 minutes with rotation at room temperature before 20 U of Hirudin (Sigma) was added to stop the cleavage reaction. The supernatant was passed through a Micro Bio spin chromatography column (Bio Rad Laboratories) and incubated with 30 µl of GT-resin for 1 hour with rotation at 4°C to remove any remaining GST. The supernatant was collected and incubated with an equal volume of either GDP- or GTP-loading buffer (3 mM GTP or GTP, 4 mM DTT, 30 mM EDTA in Binding Buffer) for 90 minutes at room temperature. The loading reaction was quenched by the addition of MgOAc to a final concentration of 30 mM and incubated at 4°C for 15 minutes. The loaded Ran was then aliquoted and stored at –70°C.

2.11.3 Ran Binding

Kap104-GST or kap104*355-8-GST was isolated as above and lysates were incubated with 30 µl of GT-resin at 4°C for 1 hour with rotation. Bound complexes

were washed three times with 1X Transport Buffer and once with Binding Buffer. Thirty µl of Ran loaded with either GTP or GDP or 30 µl of GTP-loading buffer was added to the immobilized GST fusions. Samples were incubated at room temperature for 30 minutes with rotation. The GT-resins were pelleted and the supernatants (Unbound Fraction) were passed through a Micro Bio spin chromatography column (Bio Rad Laboratories) to remove any resin. The resin-bound fusions (Bound Fraction) were washed 3 times with Binding Buffer and resuspended in 30 µl of Binding Buffer. Three-times SDS sample buffer was added to all samples, which were then heated for 15 minutes at 65°C before separation by SDS-PAGE and visualization with Coomassie Blue.

2.11.4 Ran Elution

Kap104-GST or kap104*355-8-GST was complexed with Ran-GTP as described above. The Unbound fractions were collected and the resin-bound complexes washed as above. Ran-GTP was eluted from the immobilized fusions with increasing concentrations of $MgCl_2$. One ml of solution was used for each elution, except the 2 M $MgCl_2$ elution, where 500 µl were used. The final elution was done using 1 ml of 0.5 M acetic acid. All samples collected were brought to a final volume of 1 ml and 100 µl of 0.15% sodium deoxycholate and 100 µl of 100% TCA were added. Samples were mixed by inversion and incubated at 4°C overnight. The samples were subjected to centrifugation at 11,250 x *g* for 30 minutes and the supernatants discarded. The pellets were resuspended in 100 µl of water and 900 µl of acetone. Samples were incubated at –20°C for 2 hours before centrifugation at

11,250 x *g* for 30 minutes at 4°C. The supernatant was discarded and excess moisture removed by vacuum centrifugation. The pellets were resuspended in 12 µl each of Magic A and Magic B before being heated for 15 minutes at 65°C. Polypeptides were separated by SDS-PAGE and the presence of Ran detected by immunoblotting with Ran specific antibodies and ECL.

2.11.5 Nab2p Binding

Lysates of the recombinant proteins Nab2-GST, Kap104-GST, kap104*355-8-GST and GST, were incubated with 30 µl of GT-resin for 1 hour at 4°C with rotation. The Nab2-GST resin was washed 3 times with 1X Transport Buffer before incubation for 10 minutes at 37°C in 2 mM GTP, 10 mM $MgSO_4$, 50 mM Tris pH 7.4 to remove a contaminating 70 kD polypeptide. After the incubation the resin was washed 3 times as above and resuspended in 30 µl of 1X Transport Buffer. Nab2-GST was cleaved from the resin by the addition of 3 U of Thrombin and incubated at room temperature for 15 minutes with rotation. The addition of 1 U of Hirudin was used to stop the cleavage reaction. The supernatant was then incubated with 10 µl GT-resin for 30 minutes at 4°C with rotation to remove any remaining GST. The cleaved Nab2p was passed through a Micro Bio spin chromatography column (Bio Rad Laboratories) to prevent any resin from being transferred to the immobilized recombinant proteins. Kap104-GST, kap104*355-8-GST and GST were washed 3 times with 1X Transport Buffer and incubated with the cleaved Nab2p for 1 hour at 4°C with rotation to allow complexes to form. After the incubation the supernatants (Unbound Fraction) were collected and 3X SDS sample buffer added. The resin

(Bound Fraction) was washed 3 times with 1X Transport Buffer, resuspended in 30 µl and 3X SDS sample buffer added. Samples were heated at 65°C for 15 minutes and polypeptides separated by SDS-PAGE and visualized with Coomassie Blue.

2.11.6 Nab2p Elution

Kap104-GST and kap104*355-8-GST was complexed with purified Nab2p as described above. The Unbound fractions were collected and the resin-bound complexes washed as above. Nab2p was eluted from the immobilized fusions with increasing concentrations of $MgCl_2$. One ml of solution was used for each elution, except the 2 M $MgCl_2$ elution, where 500 µl were used. The final elution was done using 1 ml of 0.5 M acetic acid. All samples collected were brought to a final volume of 1 ml and 100 µl of 0.15% sodium deoxycholate and 100 µl of 100% TCA were added. Samples were mixed by inversion and incubated at 4°C overnight. The samples were subjected to centrifugation at 11,250 x *g* for 30 minutes and the supernatants discarded. The pellets were resuspended in 100 µl of water and 900 µl of acetone. Samples were incubated at –20°C for 2 hours before centrifugation at 11,250 x *g* for 30 minutes at 4°C. The supernatants were discarded and excess moisture removed by vacuum centrifugation. The pellets were resuspended in 12 µl each of Magic A and Magic B before being heated for 15 minutes at 65°C. Polypeptides were separated by SDS-PAGE and visualized by silver stain.

2.11.7 Ran Mediated Release of Kap104 and kap104*355-8

Lysates containing the recombinant proteins Nab2-GST, Kap104-GST and kap104*355-8-GST were incubated with 90 µl of GT-resin for 1 hour at 4°C with rotation. Kap104-GST and kap104*355-8-GST were washed three times with 1X Transport Buffer and the resin was resuspended in 90 µl of 1X Transport Buffer. The fusions were cleaved by incubation with 12 U of Thrombin for 1 hour at room temperature with rotation. The cleavage reaction was stopped by the addition of 6 U of Hirudin. The supernatants were passed through a Micro Bio spin chromatography column (Bio Rad Laboratories) to remove any resin and incubated with 20 µl GT-resin for 1 hour at 4°C with rotation to remove any remaining GST. Nab2-GST was washed 3 times with 1X Transport Buffer, incubated for 10 minutes at 37°C in 2 mM GTP, 10 mM $MgSO_4$, 50 mM Tris pH 7.4 to remove a contaminating 70 kD polypeptide and washed 3 times with 1X Transport Buffer. The cleaved Kap104p and kap104*355-8p were passed through a Micro Bio spin chromatography column (Bio Rad Laboratories), added to immobilized Nab2-GST and incubated for 1 hour at 4°C with rotation to form complexes. After the complexes had formed they were washed 3 times with 1X Transport Buffer and once with 1X Binding Buffer. The samples were split into three equal volumes and Ran-GTP, Ran-GDP or GTP Loading Buffer added. Samples were incubated for 30 minutes at room temperature with rotation and the supernatant was passed through a Micro Bio spin chromatography column (Bio Rad Laboratories) (Released Fraction). The resin-bound complexes were washed three times with 1X Binding Buffer and resuspended in 30 µl. The fusions were cleaved by incubation with 3 U Thrombin of for 15 minutes at room temperature with rotation. The cleavage reaction was stopped by the

addition of 1 U of Hirudin. The supernatants were passed through a Micro Bio spin chromatography column (Bio Rad Laboratories) (Bound Fraction) and 3X SDS sample buffer added. Samples were heated for 15 minutes at 65°C before separation by SDS-PAGE and visualization with Coomassie Blue.

2.12 GFP Analysis

W303 haploid cells containing Kap104-pYES2 or kap104*355-8-pYES2 and a plasmid borne copy of Nab2-GFP were grown in 2 ml of SM containing 2% glucose at 30°C overnight. The cultures were used to inoculate 50 ml of fresh media to produce a culture at early logarithmic phase after growth overnight. Cells were washed twice with sterile water and placed into 50 ml SM containing 2% raffinose to grow for 2 hours as above. After growth 6 ml were removed and used as the zero time point and replaced with 5 ml of 20% galactose and 1 ml of SM with no carbon source. After the addition of galactose 6 ml were removed at the indicated time points to check GFP (green fluorescent protein) localization and determine expression levels of Kap104p or kap104*355-8p. One ml was used to detect GFP chimeras by fluorescent microscopy (Zeiss Axioskop 2 fixed with a Spot camera, Diagnostic Instruments Inc., Sterling Heights, MI). The remaining 5 ml were used to monitor the levels of kap104*355-8p or Kap104p chimeras as described above (Section 2.10).

2.13 Immunofluorescence Microscopy

W303 haploid cells containing Kap104-prA-pYES2 or kap104*355-8-prA-pYES2 were grown overnight in 20 ml of SM containing 2% glucose at 30°C.

Cultures were placed into 300 ml of fresh media and allowed to grow at 30°C to early logarithmic phase. Cells were collected by centrifugation at 3,000 x *g* for 10 minutes at room temperature and washed several times with sterile water. Cells were transferred to 300 ml of SM containing 2% raffinose to grow for 2 hours at 30°C and 50 mls of culture were removed as the zero time point. The 50 ml of culture was replaced with 30 ml of 20% galactose and 20 ml of SM and the culture was placed back at 30°C to grow. Fifty ml of culture were taken at the indicated times and processed as described below. Five ml of culture were used to detect the induction of kap104*355-8-prA and Kap104-prA chimeras as described above (Section 2.10) the remaining 45 ml were used for immunofluorescence. The 45 ml were subjected to centrifugation at 2,200 x *g* for 5 minutes to pellet the cells and the supernatant discarded. The cells were resuspended in fixing solution (3.7% (v/v) formaldhyde, 1X phosphate buffer) and incubated at room temperature for 4 minutes or 8 minutes. After the indicated times cells were pelleted by centrifugation at 2,000 x *g* for 1 minute, supernatants discarded and the cells resuspended in 500 µl of 1X Phosphate Buffer (100 mM KH_2PO_4 pH 6.8, 37.4 mM KOH, 0.5 mM $MgCl_2$) to stop the fixation. Cells were washed again as above and resuspended in 1 ml of sorbitol citrate (100 mM KH_2PO_4, 36.4 mM Citric Acid, 833 mM sorbitol, 0.5 mM $MgCl_2$). DTT was added to 1 mM final concentration and samples incubated at room temperature for 10 minutes. Cells were pelleted as above, resuspended in a minimal volume of sorbitol citrate, and 0.1% (v/v) of glusulase (NEN), 10 µg of zymolyase 20T/ml (Sigma) and 10 µg of mutinase/ml (Sigma) added to digest cell walls. Cells were incubated at 30°C for 60-90 minutes with occasional gentle mixing. Cells were

digested until approximately 90% of cells were phase dark in water. Enzymes were removed by washing the cells gently with sorbitol citrate two times and resuspended in a minimal volume of sorbitol citrate. One µl of cell suspension was mixed with 20 µl of sorbitol citrate and spotted on multiwell slides coated with poly-L-lysine. After 5 minutes at room temperature the excess solution was removed and slides placed in methanol for 5 minutes to fix cells to the slide. Slides were then taken from the methanol and placed into acetone for 30 seconds before they were blocked with PBS-T (40 mM K_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 15 mM NaN_3, 0.1% Tween 20) containing 2% BSA (bovine serum albumin) for 5 minutes. Slides were then incubated in a moist environment with the appropriate primary antibody at 4°C overnight. Unbound antibody was removed by washing several times with 2% BSA/PBS-T. Slides were incubated with the secondary antibody in a moist environment for 2 hours in the dark at room temperature and washed several times with 2% BSA/PBS-T before mounting with 90% glycerol, 0.2% phenylenediamine, and 0.025% diamidino-2-phenylindole dihydrochloride (DAPI) in 0.1X PBS. A coverslip was placed on the sides and sealed with nail polish. Immunofluorescence was detected with a fluorescent microscope (Zeiss Axioskop 2) and images captured using a Spot camera (Diagnostic Instruments Inc., Sterling Heights, MI).

2.14 Sub-Cellular Fractionation

2.14.1 Isolation of Cytosol and Nuclei

W303 haploid cells containing Kap104-prA-pYES2 or kap104*355-8-prA-pYES2 were grown at 30°C in 20 ml of SM containing 2% glucose overnight and

used to inoculate 4 L of fresh media. Cultures were grown overnight at 30°C to early logarithmic phase. Cells were pelleted by centrifugation at 3,000 x *g* for 10 minutes at room temperature and the supernatant discarded. Cells were washed extensively with sterile water before being placed into 4 L of SM containing 2% raffinose. After 2 hours at 30°C, 200 ml of 20% galactose were added to the cultures and they were left at 30°C for an additional 3.5 hours. Cells were then pelleted as above, washed twice with water and the pellet weighed. The pellets were resusupended in ice-cold 10 mM DTT and 100 mM Tris (pH 9.5) and incubated on ice for 10 minutes. Cells were pelleted by centrifugation at 2,200 x *g* for 5 minutes at 4°C and resuspended in 2 ml/g of pellet of ice cold 1 M Sorbitol. Cells were pelleted and resuspended in the same way and 0.1% (v/v) of glusulase, 10 μg of zymolase 20-T/ml and 10 μg of mutinase/ml added. Samples were incubated at 30°C with gentle agitation for ~3 hours or until the majority of cells were spheroplasts. Cells were then pelleted by centrifugation at 1,350 x *g* for 10 minutes at 4°C and the supernatant discarded. Cells were washed with ice-cold 1.1 M sorbitol and resuspended in 1 ml/g of pellet of ice-cold 1.1 M sorbitol with 0.001 solution P added. The samples were subjected to centrifugation through a 7.5% Ficoll 400/1.1 M sorbitol cushion at 2,500 x *g* for 20 minutes at 4°C. The supernatants were discarded and 2 ml/g of pellet of ice-cold 8% PVP (polyvinylpyrrolidone) added with 0.001 solution P (400 μg/ml Pepstatin A, 18 mg/ml PMSF). Samples were homogenized with a polytron (Polytron Inc., Tallahassee, FL) for 30 seconds 3 times at level 6 and an equal amount of 0.6 M sucrose/8% PVP with 0.001 solution P was added. Samples were mixed well by homogenization or vortex and subjected to centrifugation at 9, 200 x *g* for 20 minutes

at 4°C to pellet the crude nuclei. (This crude nuclear pellet was used for immunopercipations as discussed below, Section 2.14.2.) The supernatants from this centrifugation were stored at –70°C and labeled the cytosolic fraction. The pellets of crude nuclei were resuspended in 5 ml of 2 M sucrose/8% PVP and 0.001 solution P by polytron as described above. Once resuspended an equal volume of 2.25 M sucrose/8% PVP with 0.001 Solution P was added and the samples mixed with the polytron as above. The samples were layered onto the proper number of centrifuge tubes containing a 3 step sucrose gradient. The gradients were made with 2.15 M sucrose/8% PVP, 2.25 M sucrose/8% PVP and 2.52 M sucrose/8% PVP. Once loaded the samples were subjected to centrifugation at 103,500 x *g* for 4 hours at 4°C. The purified nuclei were extracted from the 2.25 M and 2.52 M interface and stored at –70°C.

2.14.2 Nuclear Envelopes

Crude nuclei, isolated as above, were quantified by measuring the OD_{260} of a 10 µl aliquot of nuclei in 990 µl of 1% SDS. 8% PVP was added to the nuclei samples to lower the density of the samples below a refractive index of 1.430. The nuclei were then pelleted from solution by centrifugation at 113,400 x *g* for 1 hour at 4°C. The supernatants were discarded and the pellets resuspended by vortexing at 4°C in 1 ml per 100 OD_{260} of DNAse Buffer (20% (v/v) DMSO, 20 µg/ml DNaseI, 0.001 Solution P, 0.1 mM $MgCl_2$, bis-Tris-HCL pH 6.5). After the samples were fully resuspended they were left at room temperature for 5 minutes. The samples were mixed with 3 volumes of 2.1 M sucrose/20% Nycodenz and 0.001 solution P

and placed in the proper number of centrifuge tubes. The nuclei were overlaid with an equal volume of 2.0 M sucrose/bis-Tris-Mg and 1.5 M sucrose/bis-Tris-Mg. The nuclear envelopes were floated up the gradient by centrifugation at 103,500 x *g* for 24 hours at 4°C. The nuclear envelopes were then isolated from the 1.5 M and 2.0 M interface and stored at –70°C. One hundred µl were taken from the NE fractions and 240 µl 1.85 M NaOH/7.4% β-mercaptoethanol added. Samples were incubated on ice for 5 minutes then 240 µl of 50% trichloroacetic acid (TCA) was added followed by another incubation on ice for 5 minutes. The proteins were pelleted by 11,250 x *g* centrifugation for 10 minutes at 4°C. The pellet was washed with water and resuspended in 50 µl each of Magic A and Magic B. Samples were heated for 15 minutes at 65°C, separated by SDS-PAGE and analyzed by immunoblotting with rabbit IgG and ECL.

2.15 Immunoprecipitations

2.15.1 From Whole Cell Lysates

W303 haploid cells containing Kap104-prA-pYES2 or kap104*355-8-prA-pYES2 were grown at 30°C in 20 ml of SM containing 2% glucose overnight and used to inoculate 4 L of fresh media. Cultures were grown overnight at 30°C to early logarithmic phase. Cells were pelleted by centrifugation at 3,000 x *g* for 10 minutes at room temperature and the supernatants discarded. Cells were washed extensively with sterile water and placed into 4 L of SM containing 2% raffinose for 2 hours at 30°C. Two hundred ml of 20% galactose were then added to the cultures to induce the expression of Kap104-prA or kap104*355-8-prA chimeras and left at 30°C for an

additional 3.5 hours. Cells were pelleted as above and washed with water. The pellets were resuspended in 12 ml of ice-cold Lysis Buffer (20 mM Na_2HPO_4 pH 7.5, 150 mM NaCl, 0.1 mM $MgCl_2$ and 0.001 Solution P) and passed through a ice-cold French pressure cell (2000ψ; Sim-Aminco Spectromic Instruments) three times. An equal volume of ice-cold Lysis Buffer with 40% (v/v) DMSO and 2% Triton-X-100 was added and samples were incubated at 4°C for 20 minutes. Samples were then subjected to centrifugation at 4°C for 15 minutes at 18,400 x *g*. The supernatants were then subjected to centrifugation at 4°C for 90 minutes at 143,500 x *g*. During this time 150 µl of IgG sepharose was equilibrated in Lysis Buffer with 0.1% Tween20. The sepharose was added to the supernatants and the samples were incubated for 1.5 hours at 4°C with rotation. The resin was collected and washed with 25 M Elution Buffer (20 mM HEPES pH 7.5, 0.05% Tween20, 0.001 Solution P, 25 mM $MgCl_2$). Resin-bound complexes were eluted off by increasing the $MgCl_2$ concentration in the elution buffer (50 mM, 200 mM, 500 mM, 1 M and 2 M). For each elution 1 mL was used with the exception of 2 M when 500 µl was used. The final elution was done with 1 ml of 0.5 M acetic acid. All samples were brought to a final volume of 1 ml and 100 µl of 0.15% sodium deoxycholate and 100 µl of 100% TCA was added. Samples were mixed by inversion and left at 4°C overnight. Samples were subjected to centrifugation at 11,250 x *g* for 30 minutes at 4°C and the supernatants discarded. Pellets were washed in 100 µl of water and 900 µl of acetone. Samples were left at –20 °C for 2 hours before centrifugation at 11,250 x *g* for 30 minutes at 4°C. The supernatants were discarded and excess moisture removed by vacuum centrifugation. The pellets were resuspended in 12 µl each of Magic A

and Magic B before being heated for 15 minutes at 65°C. Polypeptides were separated by SDS-PAGE and visualized by silver stain.

2.15.2 From Crude Nuclei

Nup116-prA genomically tagged strain containing Kap104-pYES2 or kap104*355-8-pYES2 were grown at 30°C in 20 ml of SM containing 2% glucose overnight and used to inoculate 2 L of fresh media. Cultures were grown overnight at 30°C to early logarithmic phase. Cells were pelleted by centrifugation at 3,000 x *g* for 10 minutes at room temperature and the supernatants discarded. Cells were washed extensively with sterile water and placed into 2 L of SM containing 2% raffinose for 2 hours at 30°C. Two hundred ml of 20% galactose were then added to the cultures to induce the expression of Kap104p or kap104*355-8p chimeras and left at 30°C for an additional 3.5 hours. The following steps were done essentially the same as in Section 2.13.1 with the following changes. Complete Mini protease inhibitors (Roche) were added before homogenization with the polytron and upon addition of 0.6 M sucrose/8% PVP. Once the cytosolic fractions were obtained and stored at –70°C, the pellets from this last centrifugation step were used for immunoprecipitations. The crude nuclear pellets were frozen at –70°C before progressing. The pellets were resuspended in 10 ml of Lysis Buffer (20 mM Na_2HPO_4 pH 7.5, 150 mM NaCl, and 0.1 mM $MgCl_2$) containing a Complete Mini protease inhibitor (Roche) added. The samples were passed through an ice-cold French pressure cell three times, 10 ml of Lysis Buffer containing 40% DMSO and 2% Triton-X-100 was added and the samples incubated on ice for 10 minutes. Samples were then subjected to centrifugation for 15 minutes at 18,400 x *g* at 4°C.

The supernatants were taken and subjected to centrifugation at 143,500 x *g* for 45 minutes at 4°C. The supernatants were incubated with 150 µl of IgG sepharose slurry that had been equilibrated with Wash Buffer (20 mM Na_2HPO_4 pH 7.5, 150 mM NaCl, 0.1 mM $MgCl_2$ and 0.1% Tween-20) for 1.5 hours with rotation 4°C. The samples were subjected to centrifugation for 10 minutes at 2,200 x *g* 4°C to collect the resins and were washed twice the Elution Buffer (20 mM HEPES pH 7.5, 0.005% Tween-20 and 25 mM $MgCl_2$). Resin-bound complexes were eluted from the resin with Elution Buffer containing increasing amounts of $MgCl_2$ (50 mM, 200 mM, 500 mM, 1 M and 2 M). In each case 1 ml was used with the exception of 2 M Elution Buffer when 500 µl was used. The final elution was done with 1 ml of 0.5 M acetic acid. All samples were brought to a final volume of 1 ml and 100 µl of 0.15% sodium deoxycholate and 100 µl of 100% TCA. Samples were mixed by inversion and left at 4°C overnight. The samples were subjected to centrifugation at 11,250 x *g* for 30 minutes 4°C and the supernatant discarded. The pellets were washed in 100 µl of water and 900 µl of acetone. Samples were left at –20°C for 2 hours before centrifugation at 11,250 x *g* for 30 minutes 4°C. The supernatants were discarded and excess moisture removed by vacuum centrifugation. The pellets were resuspended in 12 µl each of Magic A and Magic B before being heated for 15 minutes at 65°C. Polypeptides were separated by SDS-PAGE and Kap104p was detected by immunoblotting.

2.16 Overlay Assays

Nuclear envelopes were isolated from W303 diploid cells as described above. Proteins were precipitated with β-mercaptoethanol/NaOH and TCA as described

above, separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 30 minutes at room temperature with 5% milk/1X Transport Buffer. The membranes were incubated at 4°C rocking with cytosol containing Kap104-prA or kap104*355-8-prA chimeras diluted 1/5 in 5% milk/1X Transport Buffer with 0.001 Solution P. The membranes were washed twice with 1X Transport Buffer at room temperature and incubated for 1 hour at room temperature with the rabbit IgG. The membranes were washed as above and incubated for 45 minutes at room temperature with the donkey-HRP. The membranes were washed as above and ECL used to visualize the fusion proteins.

2.17 Two Hybrid Interactions

Kap104-pGBT9 or kap104*355-8-pGBKT7 (containing fusions with the Gal4 DNA binding domain) were transformed into the AH109 containing Nup42-pGADT7 (containing a fusion with the Gal4 DNA activating domain). Transformants were then plated on SM medium and grown at 30°C for three days to test the interaction between Kap104p or kap104*355-8p and Nup42p. A positive control, consisting of two proteins known to interact, p53 and T-antigen, where also tested for comparison (Clonetech Laboratories Inc.).

3. Results

3.1 Generation of kap104*355-8p

Chook and Blobel (1999) determined the crystal structure of Kapβ2/Trn1, and this information has helped determine which residues of Kapβ2/Trn1 are responsible for interacting with Ran-GTP. When this information is added to information gained from deletion mutants (Pollard et al., 1996), it reveals that the carboxyl terminus is responsible for binding cargo, while the amino terminus is responsible for interacting with Ran-GTP. Of particular interest is a domain near the center of the protein, termed the L7 loop, which makes several contacts with Ran-GTP (Figure 3a) (Chook and Blobel, 1999). Because the L7 loop domain extends into the cargo-binding region, it suggests that binding to Ran-GTP causes a conformational change, resulting in cargo release. The large degree of conservation over the entire length of Kapβ2/Trn1 and Kap104p allows the knowledge learned from Kapβ2/Trn1 to be applied to Kap104p (Figure 3b). Based on this, changes were introduced to Kap104p that were predicted to interrupt its interactions with Ran-GTP, generating a mutant that potentially lacks the ability to complete several rounds of import.

Amino acid residues 332-337 of Kapβ2/Trn1 align with amino acid residues 354-359 of Kap104p with a high degree of similarity (Figure 3b). Both regions contain acidic and basic amino acid residues that help create ionic and van der Waals interactions with Ran-GTP. To disrupt these interactions, site-directed mutagenesis was used to change the appropriate coding region of *KAP104*. The amino acid

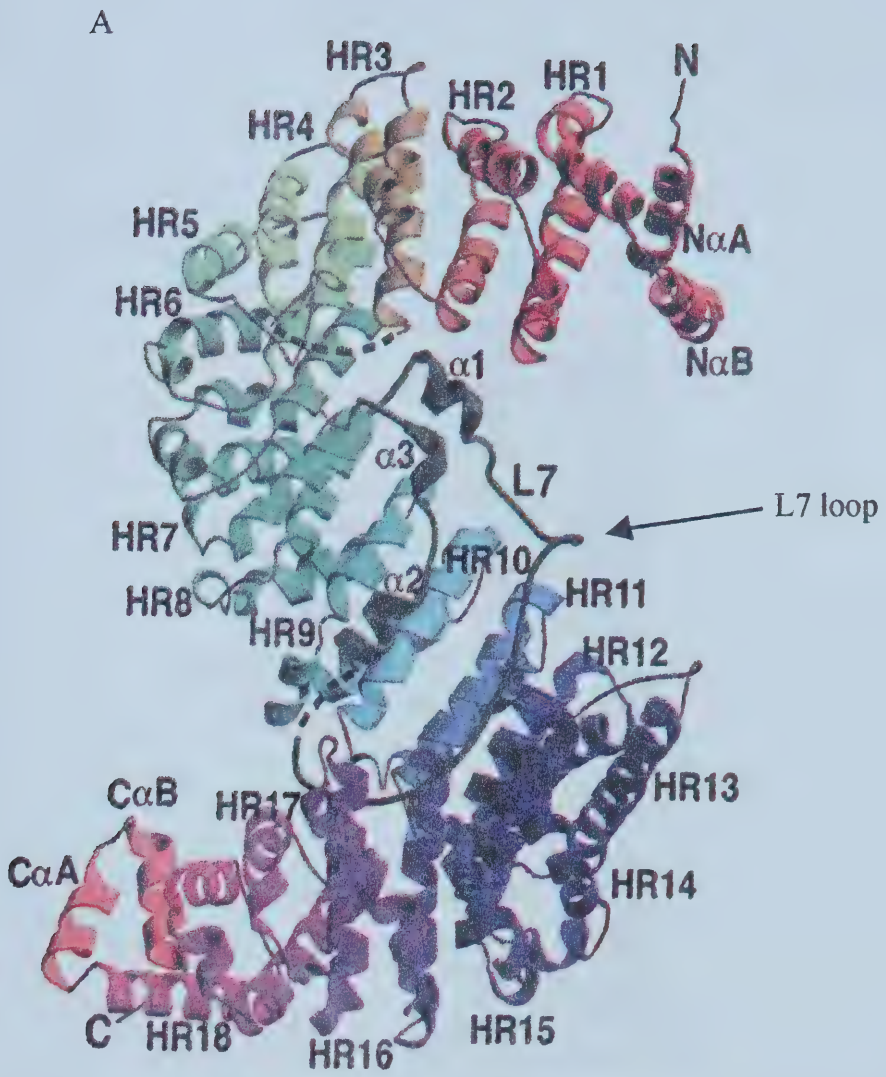
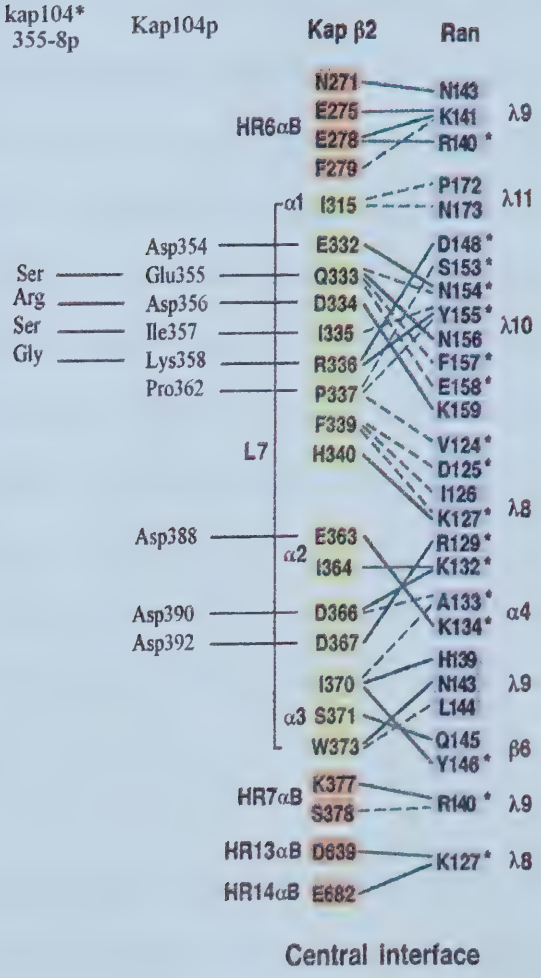


Figure 3: The crystal structure of Kapβ2/Trn1p and a closer examination of the L7 domain with the introduced changes to create kap104*355-8p.

Panel A: A ribbon diagram depicts the structure of Kapβ2/Trn1p. It consists of 18 HEAT repeats, each shown in a different colour. The L7 domain is shown by an arrow and is the site of interest. Ran interacts with Kapβ2/Trn1p at the amino terminal arch and the cargo, hnRNP A1, interacts at the carboxyl terminus. **Panel B:** Specific L7 domain amino acid side chain interactions with Ran-GTP. Dashed lines show van der Waals interactions, and ionic interactions are shown by solid lines. The corresponding amino acid residues of Kap104p are shown, as well as the changes made to create the mutant kap104*355-8p. The figures are adapted from Chook and Blobel, 1999.

B


kap104*
355-8p
Kap104p
Kap β2
Ran
N271
E275
HR6αB
E278
F279
N143
K141
R140 *
λ9
α1
I315
P172
N173
λ11
Asp354
E332
D148 *
Ser
Glu355
Q333
S153 *
N154 *
Arg
Asp356
D334
Y155 *
λ10
Ser
Ile357
I335
N156
F157 *
Gly
Lys358
R338
E158 *
Pro362
P337
K159
F339
V124 *
L7
H340
D125 *
I126
K127 *
λ8
Asp388
E363
R129 *
α2
I364
K132 *
A133 *
Asp390
D366
α4
K134 *
Asp392
D367
H139
I370
N143
λ9
L144
α3
S371
Q145
W373
Y146 *
β6
HR7αB
K377
R140 *
λ9
S378
HR13αB
D639
K127 *
λ8
HR14αB
E682
Central interface

residues believed to form ionic interactions with Ran-GTP were changed to neutral or basic residues to disrupt those interactions while neutral amino acids were changed to neutral amino acids with shorter side chains to disrupt the van der Waals interactions (Figure 3b). More specifically, the acidic residues, E355 and D356, were changed to the neutral amino acid serine and to the basic amino acid arginine, respectively. The neutral residue I357 was changed to serine, a neutral amino acid with a shorter side chain, to help destabilize the hydrophobic interactions. And finally, the basic residue, K358, was changed to a neutral amino acid, glycine, to break ionic interactions. The changes addressed above were predicted to disrupt the interactions between Kap104p and Ran-GTP but have minimal affects on the interactions between Kap104p and its substrate or nucleoporins. The proline residue (P362) was maintained in an attempt to prevent any change in the overall structure of the L7 loop domain and to prevent any inadvertent changes in the cargo-binding region. To ensure the mutant contained only the desired nucleotide changes, it was ligated into pGEM-T and the gene sequenced. All subsequent sub-cloning events used kap104*355-8-pGEM-T as a template or source.

The resulting mutant, termed kap104*355-8p, was predicted to be deficient in Ran-GTP binding but to maintain its ability to bind its cargo and nucleoporins. Therefore, when expressed in cells, kap104*355-8p was predicted to be capable of binding its cargo, Nab2p or Nab4/Hrp1p, and proceed with their import. However, we predicted that import would be halted at the Ran-GTP dependent step of

Kap104p-mediated import. Blockage at this site will help determine the role of Ran-GTP in nuclear transport and provide insight to Kap104p transport.

3.2 Ran binding ability of kap104*355-8p is not completely abolished

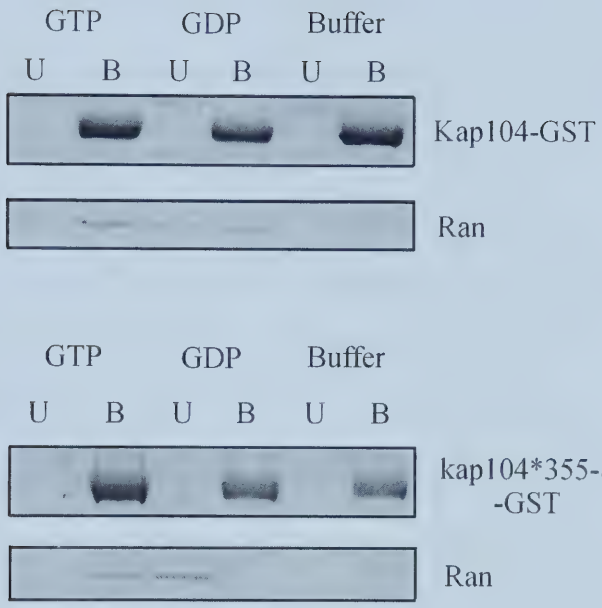
As discussed in the Introduction, the small GTPase Ran plays an essential role in nuclear transport. It has been shown to cause the dissociation of import complexes in its Ran-GTP form (Rexach and Blobel, 1995) and of export complexes in its Ran-GDP form (Fornerod et al., 1997). Work with the human ortholog of Kap104p has further explained how Ran-GTP helps dissociate import complexes. In Kapβ2/Trn1, the L7 loop domain, which extends into the cargo-binding domain, plays an important role in Ran-GTP-dependent cargo release (Chook and Blobel, 1999). When bound to Ran-GTP, the L7 loop causes a conformational change in the cargo-binding region of Kapβ2/Trn1, causing the release of the cargo hnRNP A1 (Chook and Blobel, 1999). As stated above, the changes made to make kap104*355-8p are based on its similarity to Kapβ2/Trn1 and are predicted to affect the region that interacts with Ran-GTP, rendering it unable to bind Ran-GTP.

An *in vitro* assay was used to determine if kap104*355-8p was deficient in Ran binding. Kap104-GST or kap104*355-8-GST was immobilized on a GT-resin, and Ran-GTP, -GDP or Buffer alone added. The Unbound and Bound fractions were collected, separated by SDS-PAGE and visualized with Coomassie Blue (Figure 4a). As previously shown, Kap104p interacts with both Ran-GTP and Ran-GDP (Lee and

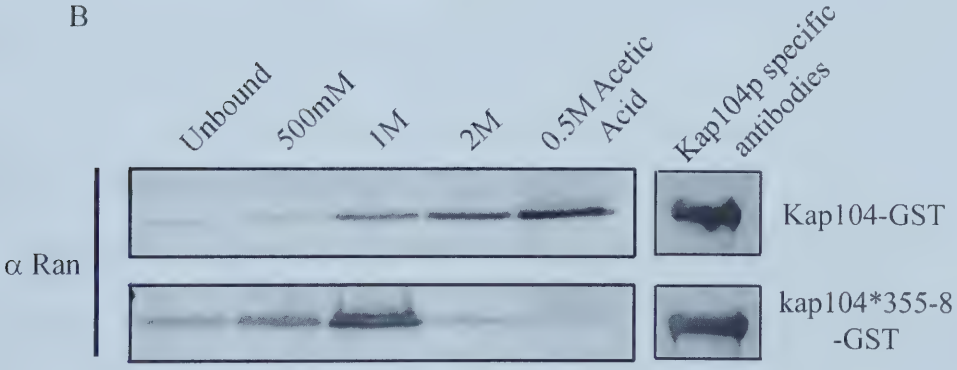
Figure 4: kap104*355-8p has weakened interactions with Ran-GTP.

Panel A: Kap104-GST and kap104*355-8-GST were expressed in *E. coli* and immobilized on GT-sepharose. Buffer, Ran-GDP or Ran-GTP was added, and samples were incubated for 30 minutes at room temperature. Unbound (U) and bound (B) fractions were collected. Polypeptides were separated by SDS-PAGE and visualized by Coomassie Blue. Both Kap104p and kap104*355-8p preferentially interact with Ran-GTP. **Panel B:** Ran-GTP bound to Kap104p and kap104*355-8p was eluted with increasing $MgCl_2$ concentrations. 0.5 M acetic acid was used for the final elution. Ran was detected with polyclonal antibodies to Ran while Kap104-GST and kap104*355-8-GST were detected with Kap104p specific antibodies, both followed by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. Note the increased salt sensitivity of the kap104*355-8p-Ran interaction.

A


GTP
GDP
Buffer
U
B
U
B
U
B
Kap104-GST
Ran
GTP
GDP
Buffer
U
B
U
B
U
B
kap104*355-8
-GST
Ran

B


Unbound
500mM
1M
2M
0.5M Acetic
Acid
Kap104p specific
antibodies
α Ran
Kap104-GST
kap104*355-8
-GST

Aitchison, 1999), but does so preferentially with Ran-GTP. Although quantitative analysis was not done kap104*355-8-GST also appeared to preferentially interacted with Ran-GTP, although its Ran binding ability was defective, as it bound less Ran-GDP. The changes introduced to create kap104*355-8p were very slight and probably resulted in disrupting the interaction with Ran but not abolishing it completely.

Since several of the ionic interactions between kap104*355-8p and Ran-GTP are presumed to have been disrupted, it was predicted that kap104*355-8p/Ran-GTP would be more sensitive to $MgCl_2$ washes than Kap104p/Ran-GTP. To test this, Ran-GTP was eluted with increasing concentrations of $MgCl_2$. The samples were analyzed by immunoblotting with a Ran specific antibody (Figure 4b). Indeed, less salt was required to cause Ran release from kap104*355-8-GST than from Kap104-GST, suggesting the ability of kap104*355-8p to interact with Ran-GTP has been compromised, presumably through altered ionic interactions.

3.3 Kap104*355-8p retains the ability to interact with Nab2p

Previous studies have shown that karyopherins mediate the import of their cargo through the use of an adaptor, as in the case of Kap60p and Kap95p (Rexach and Blobel, 1995) or by direct interaction with the cargo, as in the case of Kap104p (Lee and Aitchison, 1999). As mentioned above, the changes made to the mutant kap104*355-8p were designed to minimally affect the cargo-binding domain. To establish if kap104*355-8p was still capable of interacting directly with its cargo GST

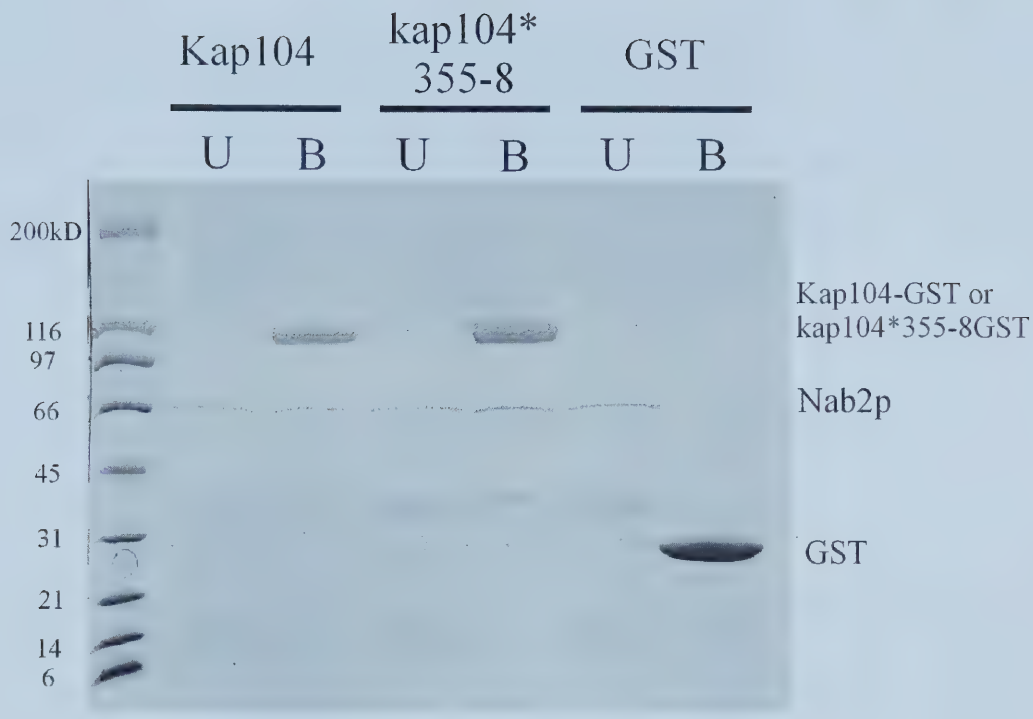


Figure 5: kap104*355-8 interacts with Nab2p.

Kap104-GST, kap104*355-8-GST or GST alone was expressed in *E. coli* and immobilized on GT-sepharose. Recombinant Nab2p was added, and the samples incubated at 4°C for 1 hour. The unbound (U) and bound (B) fractions were collected, and polypeptides were separated by SDS-PAGE and visualized by Coomassie Blue. Kap104p and kap104*355-8p both bound relatively similar amounts of Nab2p, while GST alone did not.

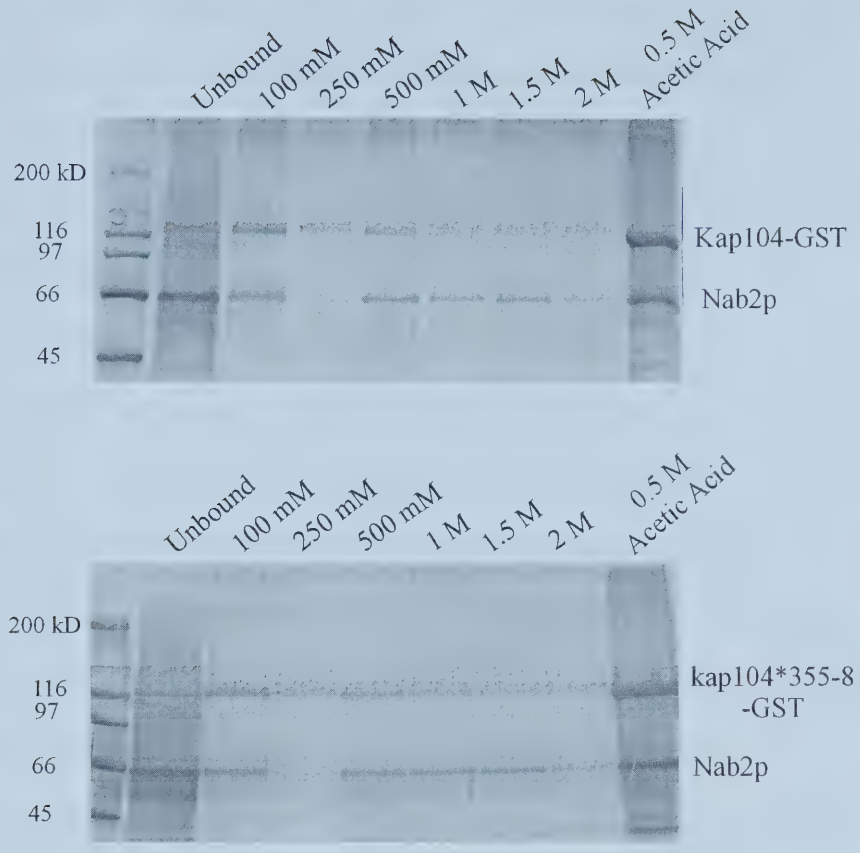


Figure 6: kap14*355-8p interactions with Nab2p appear unchanged.

Purified Nab2p bound to Kap104-GST or kap104*355-8-GST was eluted with increasing concentrations of $MgCl_2$, and a final elution with 0.5 M acetic acid. Polypeptides were separated by SDS-PAGE and visualized by silver stain. The interaction Nab2p/kap104*355-8p appears unaffected.

fusions were utilized. Kap104-pGEX2T-K, kap104*355-8-pGEX4T-1, and Nab2-pGEX2T-K were transformed into *E. coli* and expression of the fusion proteins induced with IPTG. Kap104-GST, kap104*355-8-GST or GST alone was immobilized on a glutathionine (GT) resin, and purified Nab2p added. Unbound and Bound fractions were collected, separated by SDS-PAGE and visualized with Coomassie Blue (Figure 5). Nab2p was found in the Bound fraction with kap104*355-8-GST, suggesting kap104*355-8p retained the ability to interact with Nab2p despite the changes introduced. Furthermore, the relative amounts of Nab2p bound to both kap104*355-8-GST and Kap104-GST were similar, suggesting the cargo-binding domain of kap104*355-8p was not adversely affected. Additionally, Nab2p did not bind to GST alone showing the interactions were specific for Kap104p and not occurring via the GST moiety. Furthermore, elution of Nab2p from kap104*355-8-GST and Kap104-GST with increasing amounts of $MgCl_2$ resulted in similar profiles (Figure 6), suggesting that the cargo binding domain of kap104*355-8p was not been adversely affected. The Nab2p present in the 100 mM $MgCl_2$ samples was probably a result of excess unbound Nab2p that was not completely removed, as no Nab2p is present in the 250 mM $MgCl_2$ samples.

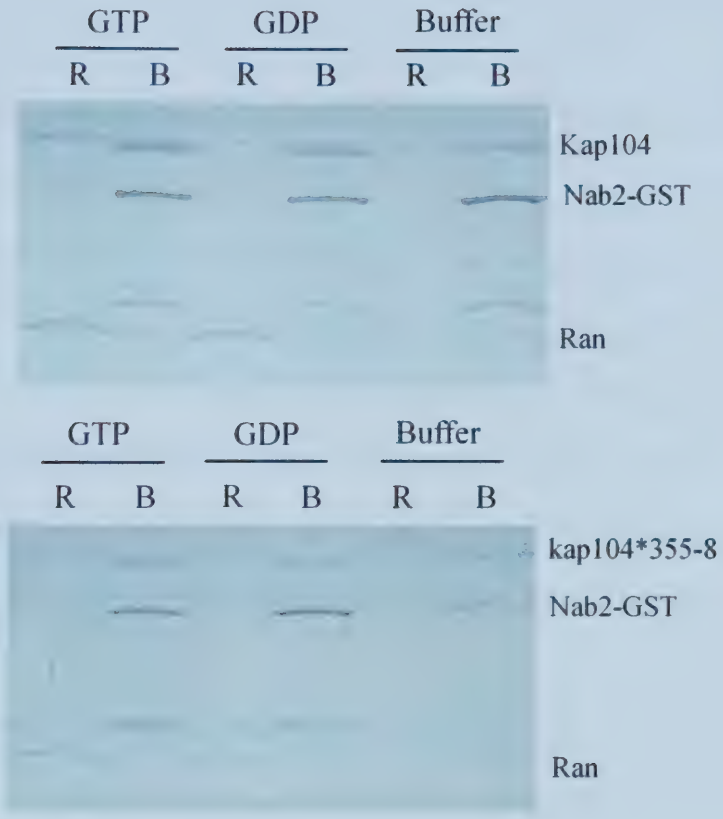
3.4 The kap104*355-8p/Nab2p complex is not dissociated by Ran-GTP

Although Ran-GTP alone causes moderate cargo release from Kap104p, Ran-GTP and mRNA are both required for efficient cargo release (Lee and Aitchison, 1999). Our results showed kap104*355-8p was defective in Ran binding, and this

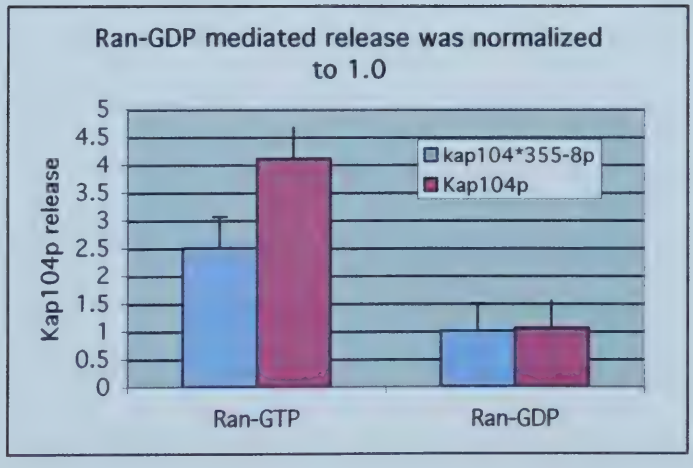
Figure 7: Ran-GTP does not dissociate the kap104*355-8p/Nab2p complex.

Panel A: Nab2-GST was expressed in *E. coli* and immobilized on GT-sepharose. Recombinant kap104*355-8p or Kap104p was added and incubated for 1 hour at 4°C. After washing, Buffer, Ran-GDP or Ran-GTP was added, and the samples were incubated for 30 minutes at room temperature. The released (R) and bound (B) fractions were collected, separated by SDS-PAGE and visualized by Coomassie Blue. Buffer and Ran-GDP caused no release of kap104*355-8p or Kap104p. The addition of Ran-GTP caused the release of Kap104p but not kap104*355-8p. **Panel B:** The results of quantitation of Kap104p or kap104*355-8 in the released fractions in 3 separate experiments are represented graphically.

A


GTP
GDP
Buffer
R
B
R
B
R
B
Kap104
Nab2-GST
Ran
GTP
GDP
Buffer
R
B
R
B
R
B
kap104*355-8
Nab2-GST
Ran

B


Ran-GDP mediated release was normalized to 1.0
Kap104p release
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
kap104*355-8p
Kap104p
Ran-GTP
Ran-GDP

raised the possibility that Ran-GTP alone may no longer be capable of causing cargo release.

To test this theory, Nab2-GST was immobilized on a GT-resin, and purified kap104*355-8p or Kap104p added. The complex was then challenged with Ran-GTP, -GDP or Buffer alone. The Released and Bound fractions were collected and separated by SDS-PAGE. When visualized with Coomassie Blue, it became evident that Ran-GTP did not cause dissociation of the kap104*355-8p/Nab2-GST complex but did cause the dissociation of the Kap104p/Nab2-GST complex (Figure 7). This suggested that the kap104*355-8p/Ran-GTP interaction had been disrupted to the point where Ran-GTP alone was no longer able to cause substrate release.

3.5 kap104*355-8p rescues *kap104-16* but not *kap104Δ*

The mutant kap104*355-8p was shown to be capable of binding Nab2p, but this interaction was not broken by the addition of Ran-GTP. Therefore, kap104*355-8p should be capable of importing its cargo to the point where Ran-GTP is required. As discussed in the Introduction, Ran-GTP is not required for a single translocation event, therefore, it is possible kap104*355-8p will be capable of one import event. However, for karyopherins to be recycled back into the cytoplasm where they can take part in further rounds of transport, it has been suggested they must interact with Ran-GTP (Schwoebel et al., 1998; Englmeier et al., 1999; Ribbeck et al., 1999). As shown above, kap104*355-8p was deficient in Ran-GTP binding and therefore presumably unable to mediate multiple rounds of import. The inability to mediate

multiple rounds of import is therefore predicted to prevent kap104*355-8p from rescuing the very slow growth and temperature sensitivity of the *kap104Δ* strain (Aitchison et al., 1996) or the temperature-sensitive strain *kap104-16* (Aitchison et al., 1996).

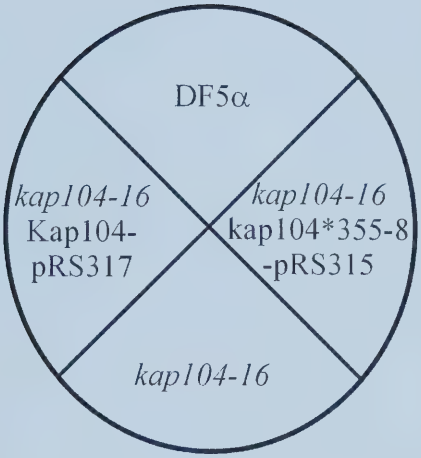
To test this theory, *KAP104*355-8* was sub-cloned into pRS315 under the control of the wild-type *KAP104* promoter and was transformed into *kap104-16* and *kap104Δ*. As a control, Kap104-pRS317, also under the control of its own promoter (Aitchison et al., 1996) was transformed into *kap104-16* and *kap104Δ*. The strains were grown for two rounds on FOA at 23°C before a third round of growth on FOA at 23°C, 30°C and 37°C. Surprisingly, kap104*355-8p was able to rescue *kap104-16* growth at the non-permissive temperature (Figure 8a). kap104*355-8p was also able to partially rescue *kap104Δ* cells at 30°C but not at 37°C (Figure 8b). As expected, the presence of Kap104p rescued growth of both strains at all temperatures. It was unexpected that kap104*355-8p was capable of rescuing *kap104-16* but not *kap104Δ*, because it has been previously shown that in *kap104-16* cells, Kap104p is quickly degraded at the non-permissive temperature (Aitchison et al., 1996). However, it is possible that a small amount of Kap104p remains, and that it works cooperatively with kap104*355-8p to complete import of substrates at a slower rate, thus allowing growth at 37°C.

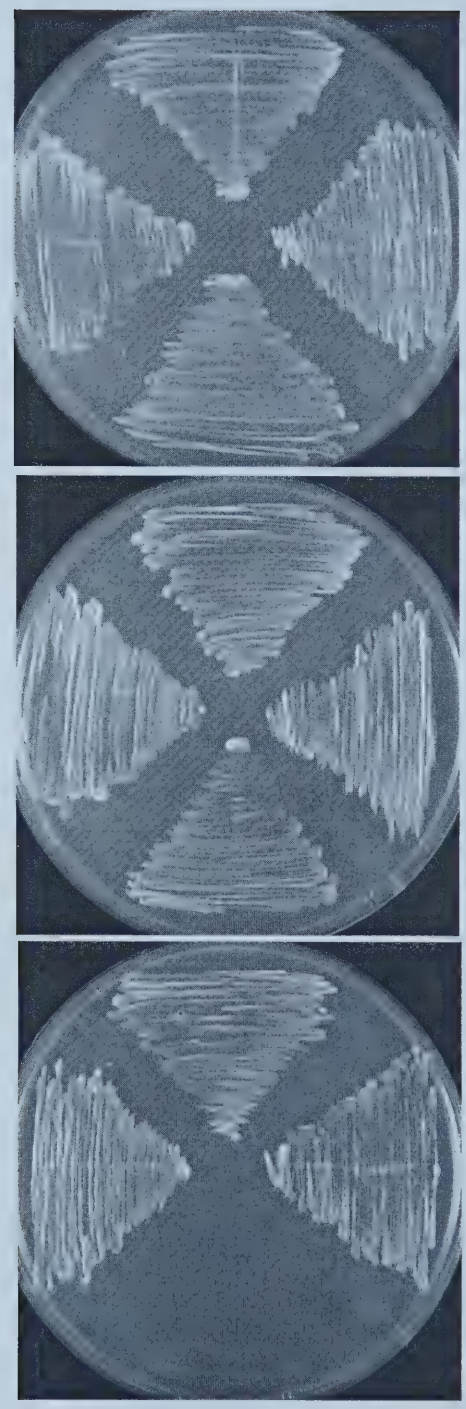
3.6 Over-production of kap104*355-8p causes a dominant negative phenotype

Figure 8: kap104*355-8p rescues *kap104-16* but not *kap104Δ*.

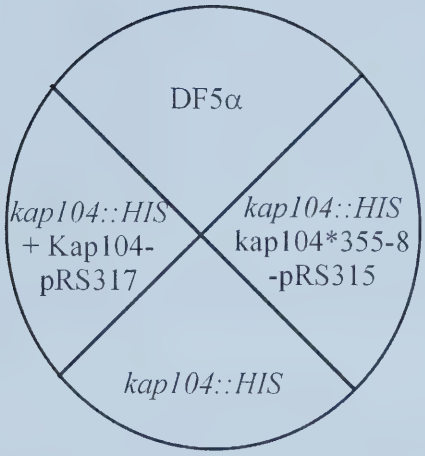
Kap104-pRS317 or kap104*355-8-pRS315 was transformed into the temperature-sensitive strain *kap104-16* (**Panel A**) or into the null strain *kap104Δ* (*kap104::HIS*) (**Panel B**).The transformants were then grown on 5-FOA at 23°C for two rounds. The cells were plated onto 5-FOA and grown for three days at 23°C, 30°C or 37°C. Kap104p rescued both strains at all temperatures, while kap104*355-8p rescued growth of *kap104-16* at 37°C and *kap104Δ* slightly at 30°C, and but not at 37°C.

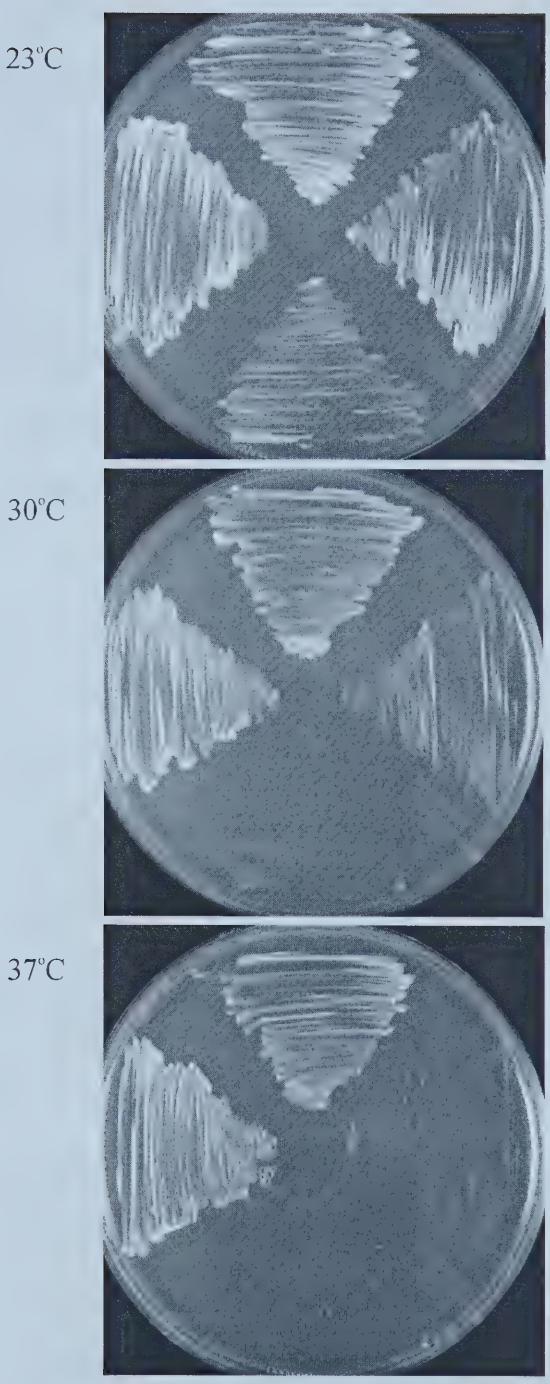
A


DF5α
kap104-16
Kap104-
pRS317
kap104-16
kap104*355-8
-pRS315
kap104-16


23°C
30°C
37°C

B

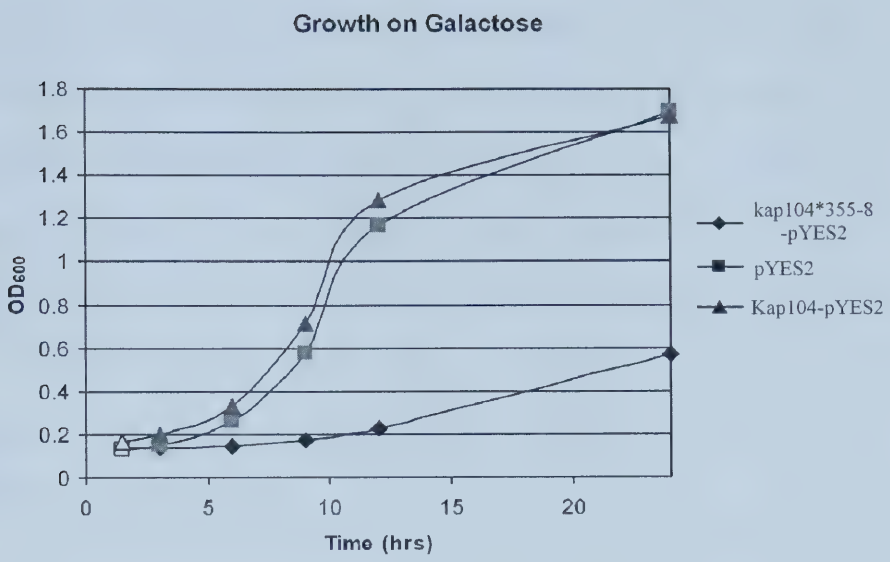

DF5α
kap104::HIS
+ Kap104-
pRS317
kap104::HIS
kap104*355-8
-pRS315
kap104::HIS


23°C
30°C
37°C

As discussed in the Introduction, Ran-GTP promotes the release of karyopherins from nucleoporins as well as the dissociation of substrates from karyopherins. If Ran-GTP is required to cause Kap104p release from nucleoporins, then kap104*355-8p is predicted to accumulate at the NPC *in vivo*. However, if Ran-GTP is required for substrate release, then expression of kap104*355-8p may bind free substrates and prevent their release in the nucleus. Since a blockage of the NPC or a lack of cargo release is proposed to cause slowed growth, an over-production of kap104*355-8p is also predicted to show a dominant negative phenotype.

To test this, *KAP104*355-8* or *KAP104* was sub-cloned into pYES2, under the control of a GAL1 promoter, and transformed into W303 haploid cells. This provided the ability of inducing the expression of *KAP104*355-8* by growing the strain on galactose. The growth of each strain was followed by obtaining the OD_{600} at the indicated times, and expression of kap104*355-8p and Kap104p was examined by immunoblot analysis. It was discovered that over-production of kap104*355-8p resulted in much slower growth than either Kap104p or pYES2 alone (Figure 9a), suggesting it had a dominant negative affect. The immunoblot analysis of samples, probed with Kap104p specific antibodies, showed similar expression levels, suggesting the difference in growth rate was a result of the mutation and not over-production of Kap104p (Figure 9b). However, it is still not clear whether the phenotype is a result of an accumulation of kap104*355-8p at the NPC blocking general transport or the lack of proper import and cargo release by kap104*355-8p.

A



B

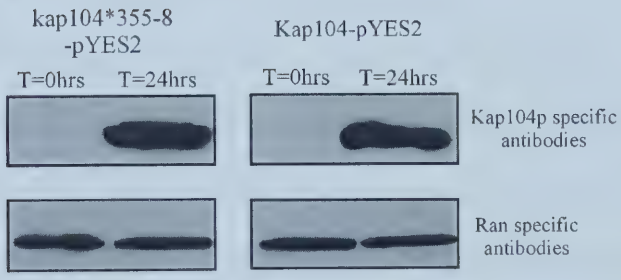


Figure 9: Over-expression of kap104*355-8p causes a dominant negative phenotype.

Panels A and **B:** Kap104-pYES2, kap104*355-8-pYES2 and empty pYES2 were grown to early log phase in SM-2% glucose and transferred to galactose to induce the expression of Kap104p and kap104*355-8p. Samples were taken at various times, and the optical density (OD_{600}) was used to follow growth (**Panel A**). Kap104p over-expression had no affect on growth, while kap104*355-8p over-expression caused slowed growth. Samples were also taken at each point to determine expression levels of Kap104p and kap104*355-8p (**Panel B**). The presence of Kap104p and kap104*355-8p was detected by immunoblotting with polyclonal antibodies. As a loading control, the level of Ran was detected with a Ran specific polyclonal antibody.

3.7 Kap104*355-8-prA accumulates at the NE by immunofluorescence

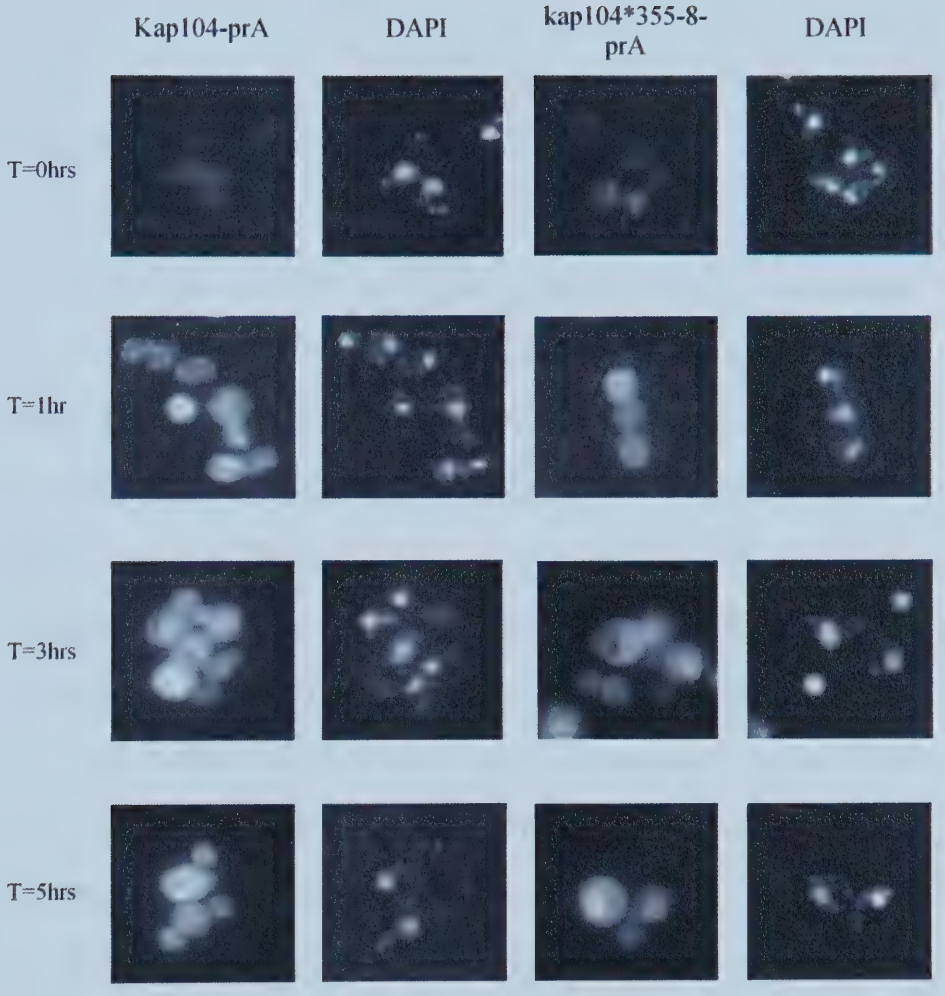
Previous studies localized Kap104p by immunofluorescence to the cytoplasm and associated with the NPC (Aitchison et al., 1996). Similar results have been discovered with other import karyopherins such as Kap123p (Rout et al., 1997). It was predicted that kap104*355-8p would have a similar localization but with an accumulation at the Ran-GTP dependent site. If binding to Ran-GTP was required at the NPC, we expected to see an increase in NPC localization. However, if Ran-GTP was required in the nuclear interior, we postulated we would see a nuclear accumulation of kap104*355-8p.

To investigate this further, the IgG binding domains of *S. aureus* protein A (prA) were sub-cloned into kap104*355-8-pYES2 and Kap104-pYES2 to create kap104*355-8-prA and Kap104-prA fusions, which could be induced by growth on galactose. At 1 hour of induction, both kap104*355-8-prA and Kap104-prA localized primarily to the cytoplasm by in-direct immunofluorescence microscopy (Figure 10a). After 3 hours of induction, the localization of kap104*355-8-prA became more pronounced at the NE, and this localization persisted to 5 hours of induction. Kap104-prA, on the other hand, remained cytoplasmic at all time points. Expression levels of kap104*355-8-prA and Kap104-prA were examined by immunoblot analysis and found to be similar, showing that the difference in localization was a result of the mutation, and not a difference in expression levels (Figure 10b). The accumulation of kap104*355-8-prA at the NE suggested the Ran-GTP dependent step of Kap104p-mediated transport was at, or within, the NPC itself.

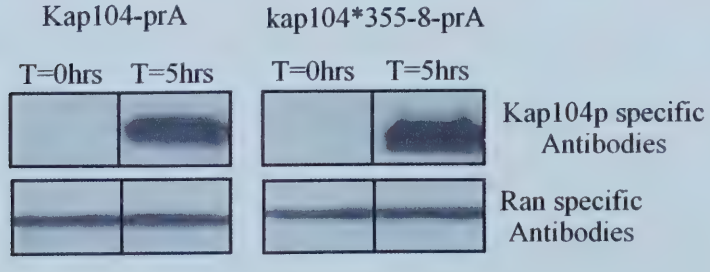
Figure 10: kap104*355-8-prA accumulates at the NE.

Panel A: W303 haploid cells containing kap104*355-8-prA-pYES2 or Kap104-prA-pYES2 were grown to early log phase on glucose, switched to raffinose/galactose and grown at 30°C to induce the expression of Kap104-prA and kap104*355-8-prA. Samples were taken at 0, 1, 3 and 5 hrs. The localizations of Kap104-prA and kap104*355-8-prA were determined by immunofluorescence, and DAPI staining identified nuclear DNA. At 3 hrs and 5 hrs kap104*355-8-prA accumulated at the nuclear envelope. Kap104-prA remained cytoplasmic at all time points. **Panel B:** The expression levels of Kap104p and kap104*355-8p were detected by immunoblotting with Kap104p specific anitbodies. Ran was used as a loading control and was detected by Ran specific antibodies.

A


Kap104-prA
DAPI
kap104*355-8-prA
DAPI
T=0hrs
T=1hr
T=3hrs
T=5hrs

B


Kap104-prA
kap104*355-8-prA
T=0hrs
T=5hrs
T=0hrs
T=5hrs
Kap104p specific Antibodies
Ran specific Antibodies

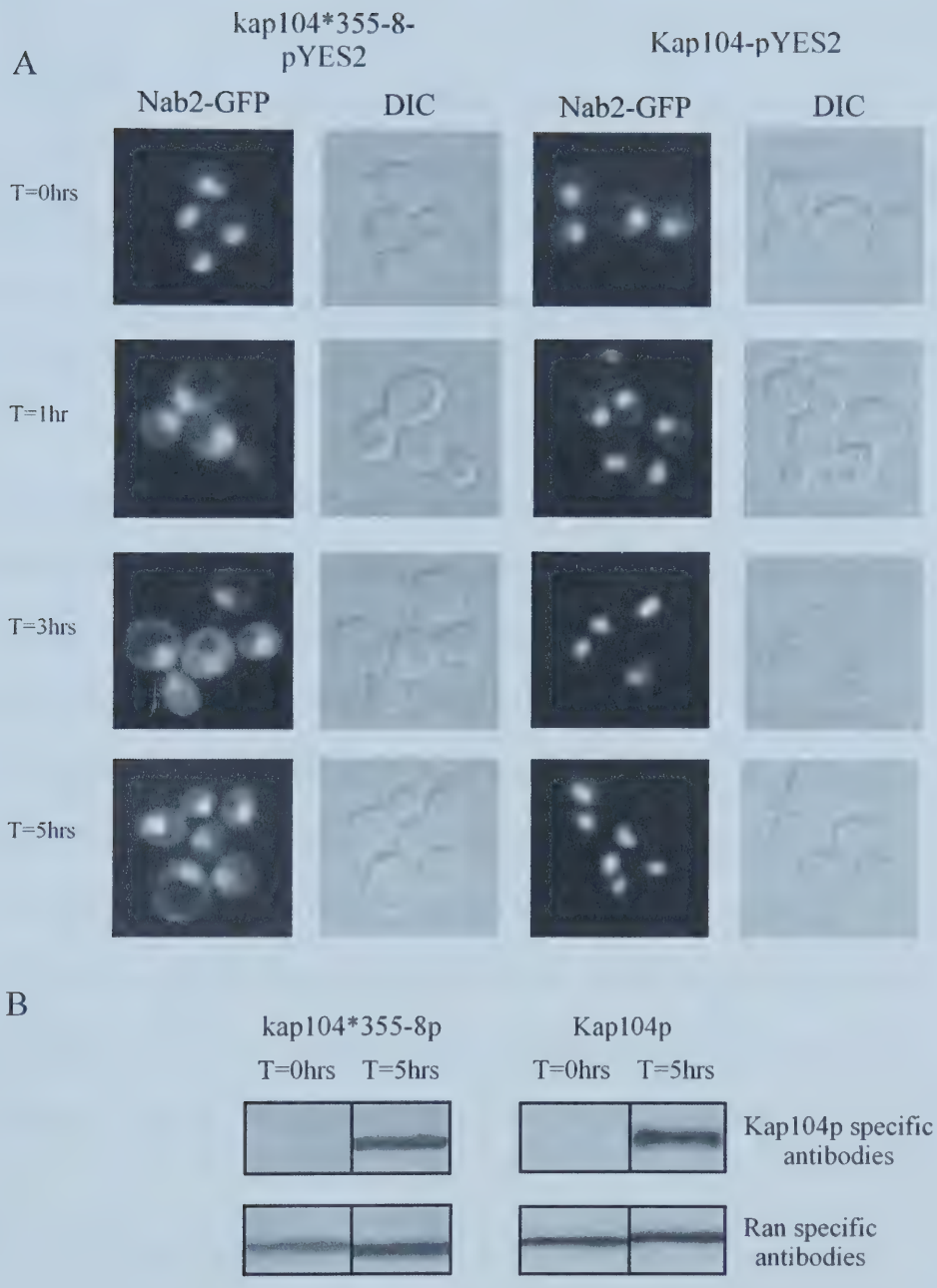
3.8 Over-production of kap104*355-8p causes Nab2-GFP mislocalization

The absence of Kap104p results in the mislocalization of Nab2p (Aitchison et al., 1996). We showed that kap104*355-8p was mislocalized when over-expressed, and therefore it was of interest to determine if the mislocalization of kap104*355-8p also resulted in the mislocalization of Nab2p.

For this purpose, kap104*355-8-pYES2 or Kap104-pYES2 was transformed into W303 haploid cells containing a plasmid-borne copy of Nab2-GFP. The expression of kap104*355-8p or Kap104p was induced by growth on galactose containing media, and the localization of Nab2-GFP determined by fluorescence microscopy (Figure 11a). At 0 hours and at 1 hour of induction, Nab2-GFP was in its steady state nuclear localization in both kap104*355-8-pYES2 and Kap104-pYES2 containing strains. After 3 hours, Nab2-GFP localization became more cytoplasmic with distinct rim staining in the kap104*355-8-pYES2 containing strain, while in the Kap104-pYES2 containing strain it remained nuclear. The difference in localization persisted even to 5 hours of induction, although there was an increase in the nuclear localization of Nab2-GFP in the kap104*355-8-pYES2 containing strain. Immunoblot analysis of samples taken at 5 hours ensured the difference in localization was a result of the mutation, not expression levels (Figure 11b). It was interesting to note that Nab2-GFP mislocalized at the same induction time as kap104*355-8-prA was shown to accumulate at the NE. The fact that Nab2-GFP mislocalized suggests Ran-GTP is required to complete Kap104p-mediated import and cargo release.

Figure 11: Over-expression of kap104*355-8p causes mislocalization of Nab2-GFP.

Panel A: Kap104-pYES2 or kap104*355-8-pYES2 was transformed into W303α cells containing a plasmid-borne copy of Nab2-GFP. Cells were grown at 30°C in SM-2% glucose to early log phase and transferred to raffinose/galactose to induce the expression of kap104*355-8p and Kap104p. Cells were observed using differential interference contrast (DIC) and GFP was detected by flourescent microscopy. The localization of Nab2-GFP was determined at 0, 1, 3 and 5 hrs. **Panel B:** The expression levels of Kap104p and kap104*355-8p were detected by immunoblotting with Kap104p specific antibodies. Ran was used as a loading control and was detected by Ran specific antibodies. The over-expression of Kap104p caused no change in Nab2-GFP localization, but over-expression of kap104*355-8p caused Nab2-GFP localization to change from the nucleus to the nuclear envelope.


A
kap104*355-8-pYES2
Kap104-pYES2
Nab2-GFP
DIC
Nab2-GFP
DIC
T=0hrs
T=1hr
T=3hrs
T=5hrs
B
kap104*355-8p
Kap104p
T=0hrs
T=5hrs
T=0hrs
T=5hrs
Kap104p specific antibodies
Ran specific antibodies

3.9 Other transport pathways are unaffected by over-production of kap104*355-8p

As discussed in the Introduction, different karyopherins import and export different substrates potentially by different pathways through the NPC. However, even though Kap104p transport is distinct from other transport pathways, if it accumulates at sites that normally contribute to other transport pathways, it could disrupt these pathways.

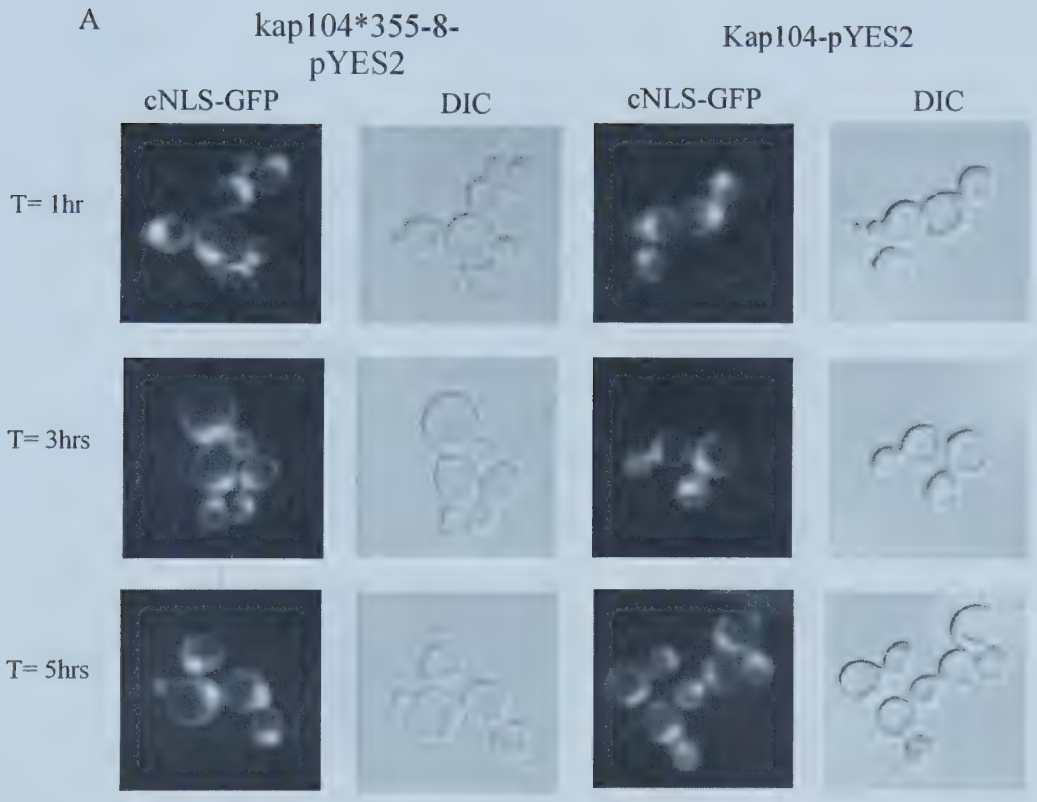
Thus, kap104*355-8-pYES2 and Kap104-pYES2 were transformed into W303 haploid cells containing plasmid-borne copies of cNLS-GFP or NES-GFP. When over-production of kap104*355-8p or Kap104p was induced by growth on galactose, the localization of cNLS-GFP or NES-GFP was examined. Import and export did not appear to be affected by kap104*355-8p or Kap104p over-production, as there was no mislocalization of the cNLS-GFP or the NES-GFP (Figure 12a and c). Immunoblot analysis showed that kap104*355-8p and Kap104p were both expressed at relatively similar levels (Figure 12b and d). These results suggested the dominant negative affect seen by kap104*355-8p over-expression was not caused by disrupted general transport but specifically by disrupted Kap104p import.

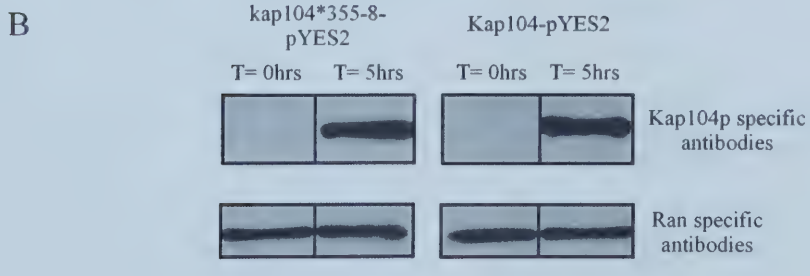
3.10 Kap104*355-8-prA enriches with NEs

While karyopherins are known to associate with NPCs, it is believed that the association is transient, occurring only while the karyopherins are transporting cargo across the NE. This becomes evident when looking at the localization of Kap104-

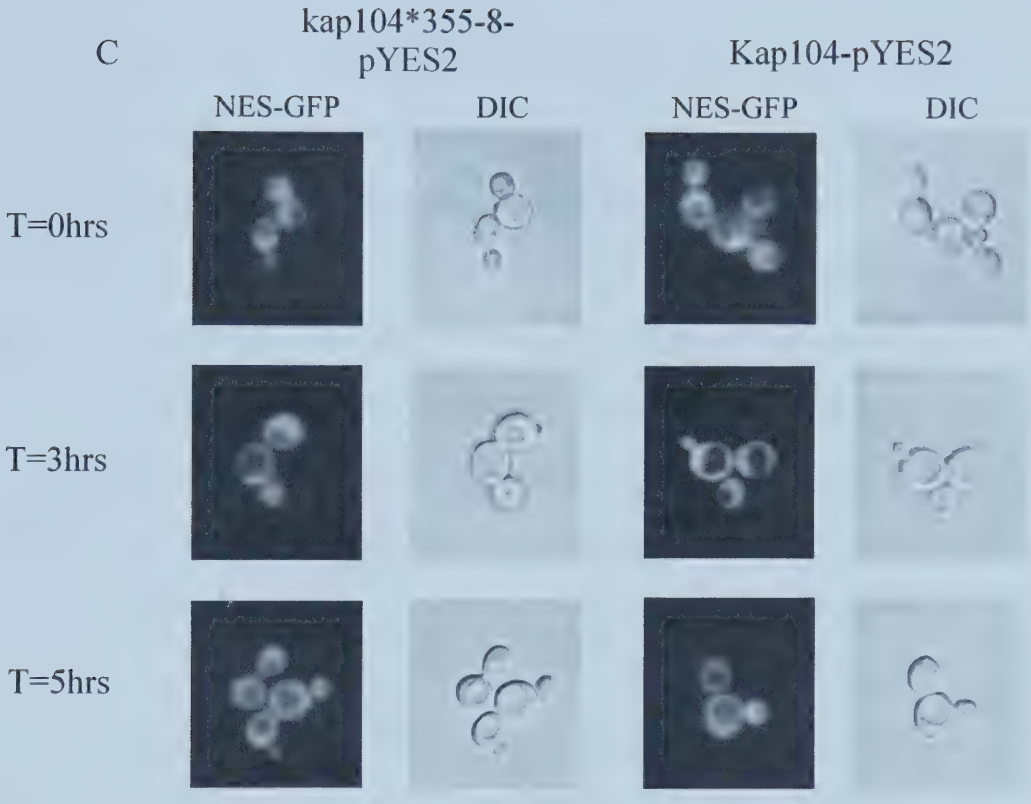
Figure 12: The dominant negative phenotype is specific to the Kap104p pathway.

Panels A and **C:** Kap104-pYES2 or kap104*355-8-pYES2 were transformed into W303α cells containing plasmid borne copies of cNLS-GFP or NES-GFP. Cells were grown to early log phase in SM-2% glucose and switched to raffinose/galatose to induce the expression of kap104*355-8p and Kap104p. Cells were observed using differential interference contrast (DIC) and GFP was detected by flourescent microscopy 1 hr, 3 hrs and 5 hrs after the addition of galactose. **Panels B** and **D**: The expression levels of Kap104p and kap104*355-8p were detected by immunoblotting with pKap104. Ran was used as a loading control and was detected by Ran specific antibodies. As shown in **Panel A,** the over-expression of Kap104p or kap104*355-8p caused no change in cNLS-GFP. Additionally, as shown in **Panel C,** no change in NES-GFP localization was observed in kap104*355-8p or Kap104p over-expression.

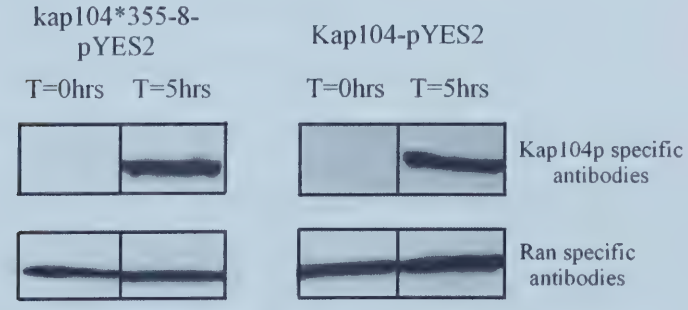

A
kap104*355-8-pYES2
Kap104-pYES2
cNLS-GFP
DIC
cNLS-GFP
DIC
T= 1hr
T= 3hrs
T= 5hrs


B
kap104*355-8-pYES2
Kap104-pYES2
T= 0hrs
T= 5hrs
T= 0hrs
T= 5hrs
Kap104p specific antibodies
Ran specific antibodies

C


kap104*355-8-pYES2
Kap104-pYES2
NES-GFP
DIC
NES-GFP
DIC
T=0hrs
T=3hrs
T=5hrs

D


kap104*355-8-pYES2
Kap104-pYES2
T=0hrs
T=5hrs
T=0hrs
T=5hrs
Kap104p specific antibodies
Ran specific antibodies

prA. Previous studies have shown Kap104-prA associating with NPC by immunofluorescence, but this interaction is not strong enough to cause Kap104p to enrich with NPCs by sub-cellular fractionation (Aitchison et al., 1996). Our results showed that kap104*355-8-prA accumulates at the NE, presumably because it is deficient in Ran-GTP-binding and is therefore unable to release from the NPC. As discussed in the Introduction, transport through the NPC has been suggested to occur via a series of binding and release steps (Rout et al., 2000). The mutant made in this thesis is deficient in Ran-GTP binding and therefore appears to become stuck at one of the binding sites. However, as discussed in the Introduction, Kap104p has not been reported to interact with nuclear-biased nucleoporins, suggesting the Ran-GTP dependent step is within the NPC. Therefore, we predicted kap104*355-8-prA would localize to the NE fraction.

In an attempt to further identify the Ran-GTP dependent site, kap104*355-8-prA or Kap104-prA was induced and its sub-cellular location determined. The prA chimeras were induced for 3.5 hours by growth on galactose before cells were collected for fractionation. Samples from the cytoplasmic, nuclear and nuclear envelope fractions were analyzed by immunoblotting for the presence of the prA chimeras (Figure 13). Kap104-prA was present mainly in the cytoplasmic fraction, only slightly in the nuclear fraction and absent from the nuclear envelope fraction. Kap104*355-8-prA, on the other hand, was found in all three fractions. Because kap104*355-8-prA was found in the nuclear envelope fraction, it further supports the theory that the Ran-GTP-dependent site was at the NPC instead of within the nucleus.

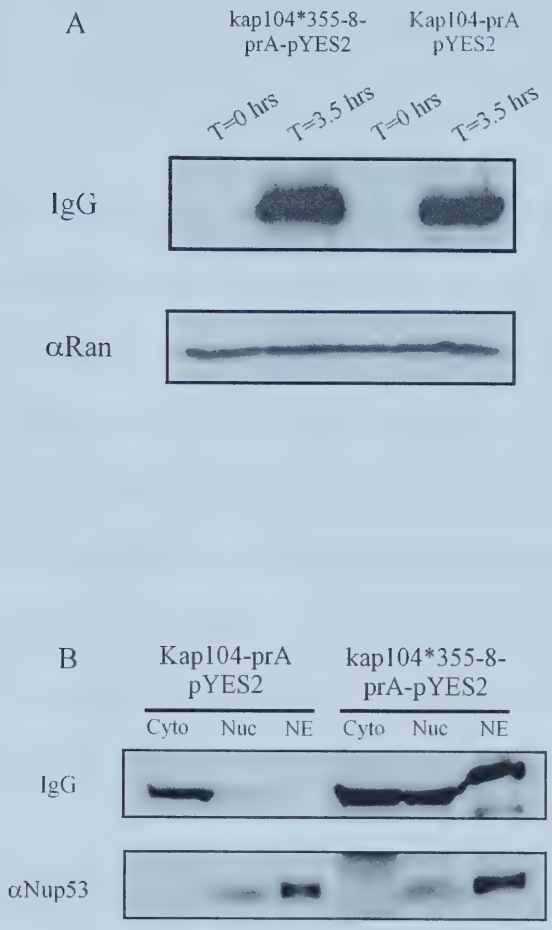


Figure 13: kap104*355-8-prA enriches with the NE sub-cellular fraction.

Kap104-prA-pYES2 and kap104*355-8-prA-pYES2 were transformed into W303α cells.
Cells were grown to early log phase in SM-2% glucose and switched to raffinose/galatose to induce the expression of kap104*355-8p and Kap104p. Samples were taken before the addition of galactose and after 3.5 hrs of growth to check for the expression of kap104*355-8-prA and Kap104-prA (**Panel A**). The fusions were detected by immunoblotting with IgG. Ran was monitored as a loading control and detected by Ran specific antibodies. Cytosol, nuclei and nuclear envelopes were isolated after the galactose induction, and the presence of kap104*355-8-prA and Kap104-prA detected by immunoblotting (**Panel B**). Nup53p was used as a loading control and detected with Nup53p specific antibodies. Kap104-prA was present mainly in the cytosol and slightly in the nuclear fraction, while kap104*355-8-prA was present in all fractions and enriched with NEs.

The presence of kap104*355-8-prA in the nuclear fraction may be a result of kap104*355-8-prA being released into the nuclear fraction during isolation.

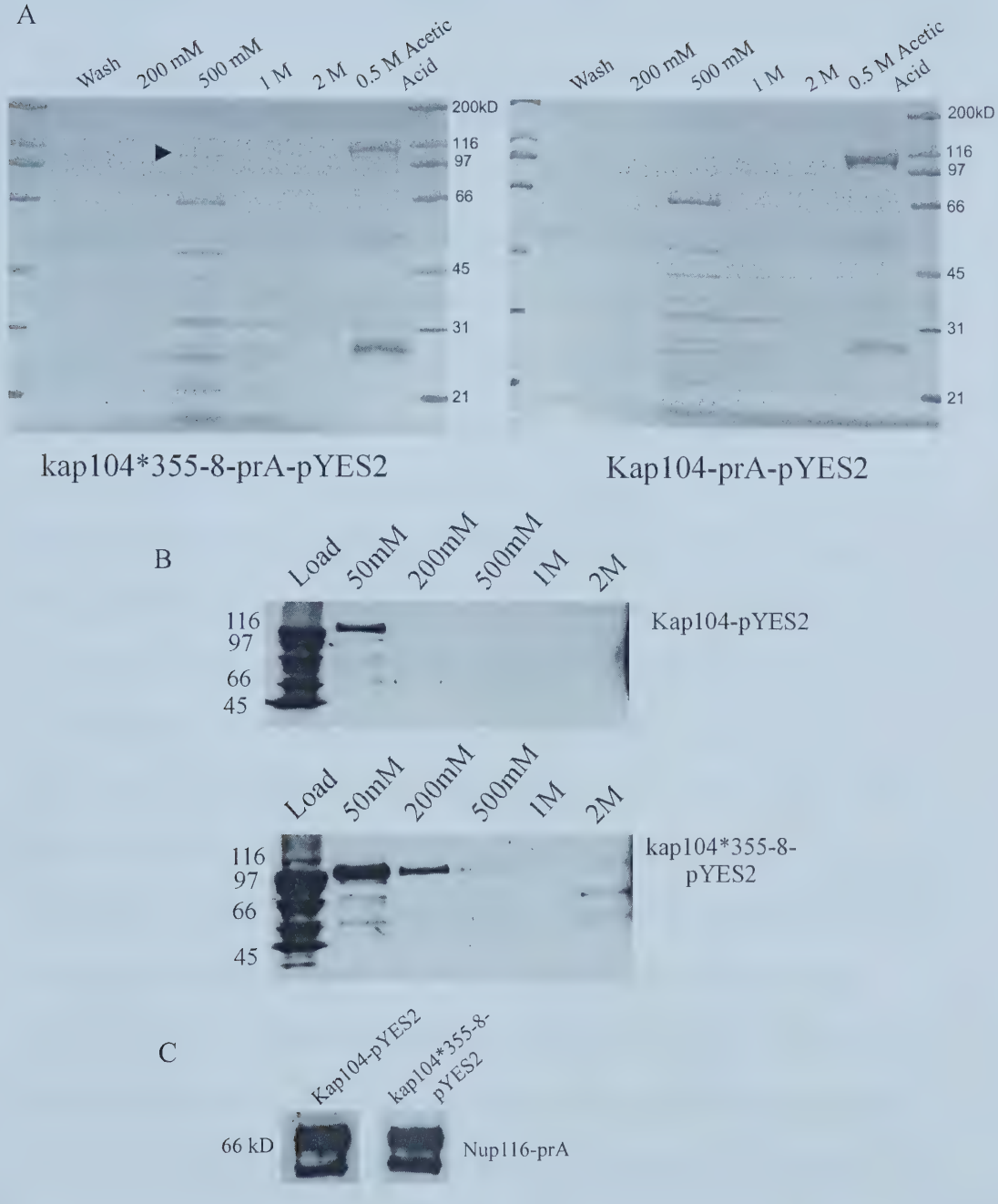
3.11 Kap104*355-8-prA interacts with Nup116p by immunoprecipitation

The association of karyopherins with FG-repeat containing nucleoporins has been previously discussed. In particular, it has been shown that Kap104p interacts directly with the FG nucleoporins Nup116p, Nup100p, Nup57p and Nup145p (Aitchison et al., 1996). Since the changes introduced to kap104*355-8p were not designed to change kap/nup interactions, it was predicted kap104*355-8p interacts with the same nucleoporins as Kap104p. Therefore, the accumulation of kap104*355-8-prA at the NE presumably results from its inability to bind Ran-GTP and release from (a subset of) these nucleoporins.

In an attempt to identify these nucleoporins, immunoprecipitations of kap104*355-8-prA or Kap104-prA were done. For this purpose, the prA chimeras were induced for 3.5 hours by growth on galactose before the cells were harvested. Cells were then lysed and the prA chimeras bound to IgG sepharose. Interacting proteins were eluted from the IgG sepharose with sequential washes containing an increasing concentration of $MgCl_2$, and samples were then separated by SDS-PAGE and visualized with Coomassie Blue (Figure 14a). A band, ~116 kD, was seen in the 500 mM $MgCl_2$ wash of kap104*355-8-prA that was not seen in the corresponding Kap104-prA sample (arrowhead, Figure 14a). Although not identified conclusively,

Figure 14: kap104*355-8-prA accumulates at Nup116p when over-expressed.

Panel A: Kap104-prA-pYES2 or kap104*355-8-prA-pYES2 were transformed into W303α cells and grown to early log phase in SM-2% glucose and switched to raffinose/galatose to induce the expression of kap104*355-8p and Kap104p. Whole cell lysates were made and the fusions immobilized to IgG sepharose. The samples were washed with increasing concentrations of $MgCl_2$ to elute interacting proteins. The final elution was done with 0.5 M acetic acid. Proteins were concentrated by TCA precipitation, separated by SDS-PAGE and visualized by silver stain. An unidentified polypeptide, ~116 kD (indicated by an arrowhead), was observed to interact with kap104*355-8-prA but not Kap104-prA. **Panel B:** Kap104-prA-pYES2 or kap104*355-8-prA-pYES2 were transformed into Nup116-prA strain and grown to early log phase in SM-2% glucose and switched to raffinose/galatose to induce the expression of kap104*355-8p and Kap104p. Crude nuclei were isolated and Nup116-prA immobilized to IgG sepharose. Interacting proteins were eluted with increasing concentration of $MgCl_2$ and concentrated by precipitation. The presence of Kap104p and kap104*355-8p were detected by immunoblotting with Kap104p specific antibodies. Kap104p is found in the low salt concentrations, while kap104*355-8p is found in the higher salt concentrations. **Panel C:** The presence of Nup116-prA was detected by immunoblotting. Similar profiles are seen, however, there is an absence of full-length Nup116-prA, presumably caused by degradation of the peptide. Thank you to Dr. T. Makhnevych for his help in doing these experiments.


A
Wash
200 mM
500 mM
1 M
2 M
0.5 M Acetic Acid
200kD
116
97
66
45
31
21
kap104*355-8-prA-pYES2
Kap104-prA-pYES2
B
Load
50mM
200mM
500mM
1M
2M
116
97
66
45
Kap104-pYES2
kap104*355-8-pYES2
C
Kap104-pYES2
kap104*355-8-pYES2
66 kD
Nup116-prA

the size of the polypeptide and known interactions between Kap104p and nucleoporins suggested the protein might be Nup116p.

To test this possibility, kap104*355-8-pYES2 or Kap104-pYES2 was transformed into a strain containing a Nup116-prA chimera integrated into the genome (Rout et al., 2000). Kap104p and kap104*355-8p were then induced by growth on galactose for 3.5 hours before the cells were harvested. Crude nuclei were extracted, lysed and the Nup116-prA chimera bound to IgG sepharose. Any interacting proteins were eluted with increasing amounts of $MgCl_2$, and samples were analyzed by immunoblotting for the presence of kap104*355-8p or Kap104p with Kap104p specific antibodies (Figure 14b). The 0.5 M acetic acid elution samples were probed for the presence of Nup116-prA to ensure equal amounts had been immobilized on the resin. Although full-length Nup116-prA was not evident, similar profiles were seen in both samples, presumably caused by the degradation of Nup116-prA (Figure 14c). While Kap104p was present only in the material eluted with 50 mM $MgCl_2$, kap104*355-8p was present in the material eluted with 50 mM, 200 mM and 500 mM $MgCl_2$. It is unclear if the presence of kap104*355-8p in the 200 mM and 500 mM elutions was a result of a higher affinity interaction with Nup116p or an increased concentration of kap104*355-8p bound to Nup116prA, which is detected *ex vivo*. Therefore, kap104*355-8p was predicted to accumulate within the NPC, filling all the binding site of Nup116p and other nucleoporins Kap104p interacts with while traversing the NPC. Although not tested, it is possible all of the kap104*355-8p could be eluted with large quantities of 50 mM $MgCl_2$.

3.12 Kap104*355-8 interacts with numerous proteins of the nuclear envelope fraction by overlay assay

Previous studies have already identified some of the nucleoporins with which Kap104p interacts *in vitro* (Aitchison et al., 1996); however, no clear indication of which nucleoporins Kap104p interacts with *in vivo* while traversing the NPC has been given. With the mutant kap104*355-8p, it became possible to determine if there was a change in the nucleoporins Kap104p interacts with while bound to Nab2p or Nab4/Hrp1p during import and while bound to Ran-GTP during export. In an attempt to identify some of the nucleoporins involved in these processes, overlay assays were done with kap104*355-8-prA and Kap104-prA.

Nuclear envelopes were isolated from W303 diploid cells, the polypeptides separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was then incubated with cytosol containing either kap104*355-8-prA or Kap104-prA. The sites where the prA chimeras interacted were then determined by a modified immunoblotting procedure (Figure 15). Interestingly, kap104*355-8-prA interacted with many more proteins than Kap104-prA, including a ~42 kD band, whereas Kap104-prA bound more avidly to a protein of ~25 kD. These data suggest one of at least three possibilities. One: the mutations introduced altered the ability of kap104*355-8p to bind proteins other than Ran, or two: Ran-GDP or -GTP present in the extract differentially modulates the ability of kap104*355-8p to interact with proteins of the NE fraction or three: the interactions are non-specific. At present, it is

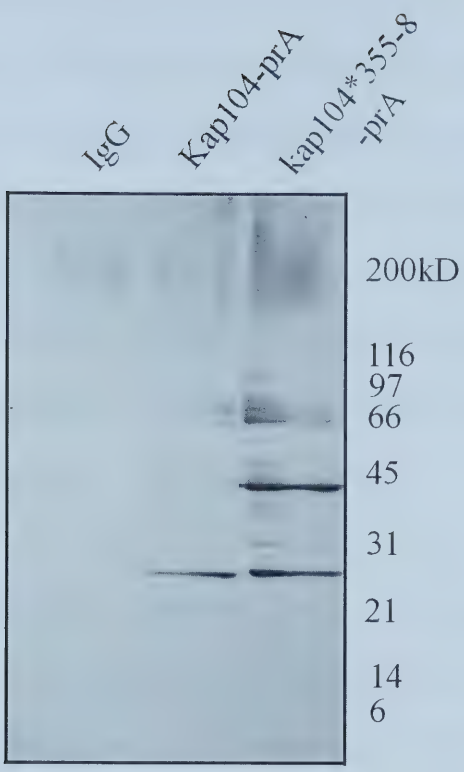


Figure 15: kap104*355-8-prA interacts with numerous proteins from the nuclear envelope fraction by overlay assay.

Nuclear envelopes were isolated from W303 diploid cells, the proteins separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then overlaid with buffer or cytosol containing kap104*355-8-prA or Ka104-prA. The membrane was then immunoblotted with IgG to identify bound chimeras. IgG alone showed no interaction bands, while Kap104-prA interacts with one protein strongly (~25 kD) and kap104*355-8-prA interacted with numerous proteins.

not possible to distinguish between these possibilities. Nevertheless, previous work in the laboratory showed the formation of the trimeric complex Kap104p/RanBP1/Ran-GDP. Although only conjecture, one intriguing possibility is that the ~25 kD band is RanBP1 and that the trimeric complex modulates Kap104p/nucleoporin interactions. Thus, if kap104*355-8p does not interact with RanBP1/Ran-GDP or –GTP as efficiently it may be free to interact with nucleoporins. It requires further study to determine if kap104*355-8p is incapable of forming this trimeric complex and to determine how the trimeric complex affects Kap104p/nucleoporin interactions.

Most of the bands visible are presumably a result of an interaction between Kap104-prA or kap104*355-8-prA and nucleoporins as nucleoporins should be the most abundant in the NE fractions used in this overlay assay. Most of these nucleoporins are predicted to be symmetrical in the NPC as Kap104p has not yet been shown to interact with more than one asymmetric nucleoporin. It is also of note that kap104*355-8-prA did not appear to interact with Nup116p by this procedure.

As mentioned above, kap104*355-8-prA interacted most strikingly with a 42 kD band. Because Nup42p is a FG repeat nucleoporin located only on the cytoplasmic face of the NPC and has been implicated directly in Crm1p mediated export, this interaction was investigated further. *KAP104* or *KAP104*355-8* was sub-cloned into the matchmaker plasmids pGBKT9 or pGBKT7, respectively. This formed a fusion between kap104*355-8p or Kap104p and the GAL4 DNA binding

domain (BD). At the same time *NUP42* was sub-cloned into pGADT7 to create a fusion with the GAL4 activation domain (AD). The constructs were transformed into AH109 cells and tested for interactions. If Kap104-BD or kap104*355-8-BD interact with Nup42-AD in the nucleus then gene activation will occur, allowing growth on selective media. It was interesting to note that while Kap104-BD was unable to grow, kap104*355-8-BD grew on selective media (Figure 16). Since the two hybrid interaction occurs in the nucleus, in the presence of Ran-GTP, presumably, only the mutant, kap104*355-8p was capable of interacting with Nup42p because it is deficient in Ran-GTP binding. This suggested Kap104p interacts with Nup42p only in the absence of Ran-GTP. The apparent sensitivity of the Nup42p/Kap104p interaction to Ran-GTP suggests that Kap104p does not bind Nup42p while it is exiting the nucleus. This possible sensitivity of the interaction to Ran-GTP however, is consistent with a role for Nup42p in Kap104p import.

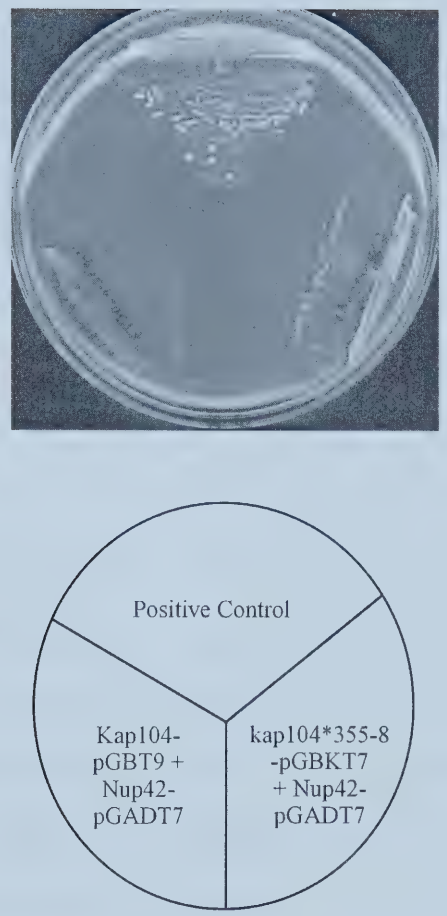


Figure 16: kap104*355-8p interacts with Nup42p by two-hybrid.

Kap104-pGBKT9 or kap104*355-8-pGBKT7 were transformed into AH109 cells containing Nup42-pGADT7 and grown on SM for 3 days at 30°C. A positive control, p53-pGKT7/ T-antigen-pGADT7, was also grown. As shown, kap104*355-8p was able to interact with Nup42p in the nucleus, allowing growth while Kap104p could not.

4. Discussion

The objective of this thesis was to gain an understanding of the role of Ran-GTP in Kap104p-mediated transport. Previous work with Kap104p identified its substrates and their novel NLSs, as well as showing the pathway to be distinct from that mediated by Kap60p/95p (Aitchison et al., 1996; Lee and Aitchison, 1999). Lee and Aitchison (1999) also showed that, in addition to Ran-GTP, mRNA was required for efficient cargo release.

Here, a mutant form of Kap104p that is deficient in Ran binding was developed by site-directed mutagenesis. The mutant retains the ability to bind Nab2p *in vitro* (Figure 5) and this interaction is stable in the presence of Ran-GTP (Figure 7). The deficiency in Ran-GTP binding results in the accumulation of kap104*355-8p (Figure 10) and Nab2-GFP (Figure 11) at the NE *in vivo*. Additionally, *ex vivo* approaches suggest that kap104*355-8p binds to Nup116p (Figure 14). These results suggest Kap104p binds Ran-GTP to release from nucleoporins and to complete the import of cargo. The lack of Kap104p import results in a dominant negative phenotype (Figure 9) and in its inability to completely rescue a *KAP104* null strain (Figure 8).

Previous experiments have shown that neither Ran nor GTP hydrolysis is required for a single translocation event (Schwoebel at al., 1998; Englmeier et al, 1999; Ribbeck et al., 1999). In particular Ribbeck et al. (1999) showed that, *in vitro,*

Ran was not required for the actual translocation of Kapβ2/Trn1, the human ortholog of Kap104p, but was required for dissociation of the import complex. The results presented here suggest that efficient Kap104p-mediated import requires an interaction with Ran-GTP *in vivo*. Presumably, the interaction with Ran-GTP facilitates Kap104p release from the NPC (Figure 17). This kinetic block was revealed by kap104*355-8p, which was deficient in its ability to bind Ran-GTP, presumably resulting in its accumulation at the Ran-GTP dependent step. The difference in Ran-GTP requirements for Kap104p import versus Kapβ2/Trn1 import could result from several factors. The most evident factor is that Kap104p is a yeast karyopherin while Kapβ2/Trn1 is a mammalian karyopherin. It is possible that evolutionary changes have occurred resulting in a different role for Ran-GTP in Kapβ2/Trn1 import. Supporting this theory is the fact that Kap104p requires both mRNA and Ran-GTP for efficient cargo release (Lee and Aitchison, 1999) while Kapβ2/Trn1 requires only Ran-GTP to cause cargo release (Siomi et al., 1997). However, the differences could also result from the *in vitro* system used by Ribbeck et al. (1999) versus the *in vivo* system used here. The addition of import components to the permeabilized cell assay used by Ribbeck et al. (1999) likely creates a concentration gradient across the NE, which may drive the translocation reactions in the absence of Ran-GTP.

The presence of asymmetric nucleoporins and the Ran gradient have been suggested to provide directionality of transport (Rout et al., 2000). For instance, an import karyopherin is proposed to have the highest affinity for nucleoporins located on the nuclear face of the NPC. At these sites Ran-GTP is predicted to ensure

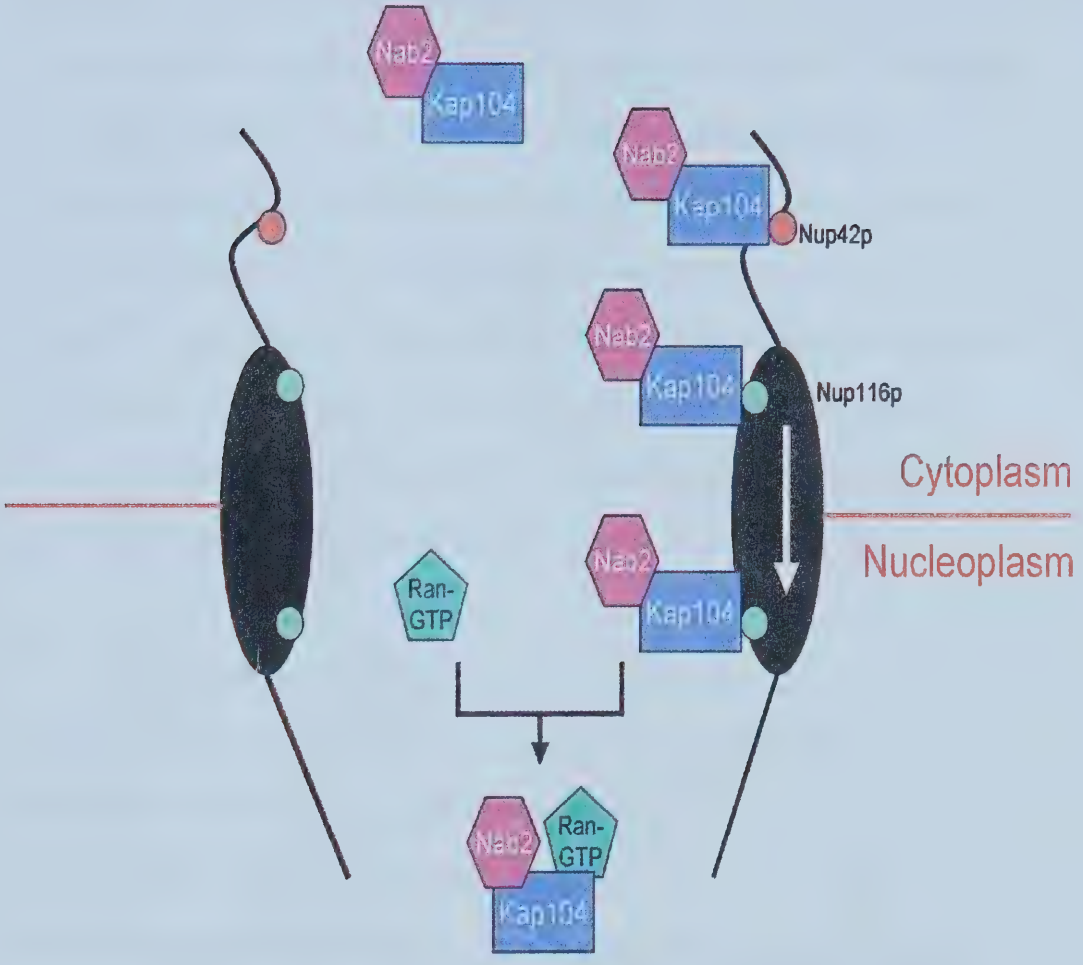


Figure 17: Model of Kap104p mediated import of Nab2p.

Kap104p possibly utilizes Nup42p as a docking site for import. Kap104p moves further into the NPC via a series of binding and release steps presumably ending at Nup116p. At this site Ran-GTP is required to release the import complex from the NPC. Although only conjecture it is possible Ran-GTP remains associated with the complex until it reaches the site of cargo release.

the import complex dissociates, leaving the substrate in the nucleus and the karyopherin free to be recycled. Supporting this theory, Kapβ2/Trn1 has been shown to interact with two nucleoporins located on the nuclear face of the NPC, Nup98p and Nup153p (Nakielny et al., 1999; Fontoura et al, 2000). Additionally, Kapβ was shown to have a higher affinity for Nup153p than for Nup358p, a nucleoporin on the cytoplasmic face of the NPC (Ben-Efraim and Gerace, 2001). However, some karyopherins, such as Kap104p, have not been detected to interact preferentially with nucleoporins at the nuclear face of the NPC (Aitchison et al., 1996; Allen et al., 2001). In these cases it is unclear if the NPC itself contributes directionality to the process or if Ran-GTP is required at the NPC to direct the reaction *in vivo*.

Results presented here suggest Ran-GTP releases Kap104p from nucleoporins. Since Kap104p has not been shown to interact with nucleoporins only located on the nuclear face it suggests that the interaction with Ran-GTP occurs within the NPC at one of the symmetric nucleoporins. Although our results suggest Nup116p may be a Ran-GTP-dependent site other nucleoporins such as Nup145p, Nup100p, or Nup57p cannot be ruled out. Localization of kap104*355-8p by immunoelectron microscopy would be useful to establish where kap104*355-8p accumulates within the NPC and may provide insight into which nucleoporins play a role in Kap104p-mediated import. A series of deletion mutants may also provide insight into which nucleoporins play a role in Kap104p-mediated import. For example, if Nup116p is the Ran-GTP-dependent step of Kap104p import it is possible that deletion of *NUP116* would remove the kinetic block, allowing kap104*355-8p to

complete import. However, it is equally possible the deletion of a different nucleoporin would have a similar affect or that a combination of deletions is required to remove this kinetic block.

The accumulation of kap104*355-8p at the NE suggests Ran-GTP may be active within the NPC and is required to release Kap104p/Nab complexes from nucleoporins. The presence of active Ran-GTP in the NPC however, poses problems for other transport pathways. For instance, Ran-GTP causes cargo release from Kap121p (Kaffman et al., 1998) and Kap60p/95p (Rexach and Blobel, 1995). This would suggest that within the NPC, active Ran-GTP would cause some import complexes to dissociate. Presumably these complexes would have to reform before continuing movement through the NPC. Work done by Ribbeck and Gorlich (2001) however, suggest that movement through the NPC is exceptionally fast and constant dissociation and association events of import complexes are not likely to occur. It is also possible that another component stabilizes those import complexes that dissociate in the presence of Ran-GTP while they traverse the NPC. Such a stabilizing factor may prevent these import complexes from dissociating within the NPC, thereby allowing their efficient transport through the NPC.

One candidate for stabilizing import complexes is RanBP1, a protein that has been shown to operate in the cytoplasm with RanGAP to promote Ran-GTP hydrolysis (Bischoff et al., 1995; Hayashi et al., 1995; Schlenstedt et al., 1997). Results from Chi et al. (1997) showed the formation of a stable complex between

Kapβ/Ran-GDP/RanBP1 and that this complex was required for efficient Kapβ import. Additionally, RanBP1 has been shown to shuttle between the cytoplasm and nucleoplasm (Plafker and Macara, 2000). Together, these results suggest RanBP1 is capable of interacting with karyopherins and provides it the opportunity to stabilize import complexes while they traverse the NPC. Similar proteins, Yrb1p or Yrb2p may play analogous roles in yeast. Similar to RanBP1, Yrb1p works with RanGAP (Rna1p in yeast) to promote Ran-GTP hydrolysis (Schlenstedt et al., 1995) and has also been shown to shuttle between the cytoplasm and nucleus (Kunzler et al., 2000) providing it opportunity to interact with karyopherins in the NPC. Although Yrb2p shows no evidence of shuttling it has been found in association with NPCs (Rout et al., 2000) also providing it the opportunity to interact with and stabilize import complexes. Therefore, in mammalian systems it is possible RanBP1/Ran-GDP interacts with karyopherins and their substrates, stabilizing the import complexes while in yeast Yrb1p or Yrb2p may perform a similar function. This system would allow Ran-GTP to be present in the NPC but not to disrupt transport complexes.

However, it is also possible that Ran-GTP acts in the NPC only on those karyopherins that require factors in addition to Ran-GTP for cargo release. In particular, Mtr10p requires RNA and Ran-GTP for cargo release (Senger et al., 1998) while Kap114p requires TATA-box containing DNA and Ran-GTP for cargo release (Pemberton et al., 1999). Supporting this theory, Mtr10p has been shown to interact with Nup116p (Allen et al., 2001) and this interaction is disrupted by the addition of Ran-GTP. The interaction between Mtr10p and Nup116p raises the possibility that

Mtr10p import may be affected by kap104*355-8p accumulation and requires further study. Although no similar nucleoporin interactions have been shown for Kap114p, it is possible that Kap114p utilizes a similar pathway through the NPC. Additionally, Kap121p has also been shown to interact with Nup116p and this interaction is disrupted by the presence of Ran-GTP (Marelli et al., 1998). Like Kap104p, Kap121p does not appear to interact preferentially with asymmetric nucleoporins. It appears that its highest affinity-binding site is at the symmetrically disposed nucleoporin, Nup53p (Marelli et al., 1998). It remains to be determeined how Ran may differentially affect this interaction and the interaction of Kap121p with its cargos.

If Ran-GTP promotes Kap104p release from nucleoporins within the NPC, as the results presented here suggest, it remains to be discovered how Kap104p moves to the nuclear interior. Although it is possible the movement occurs by diffusion it seems more probable that another factor associates with the complex and moves it to the site of mRNA transcription. One possible candidate is Nup2p, a mobile nucleoporin that shuttles between the nuclear and cytoplasmic faces of the NPC (Dilworth et al., 2001). This movement would allow Nup2p the opportunity to bind Kap104p in the NPC interior and move it further into the nucleus. Although, no association between Nup2p and Kap104p has been shown to date, Nup2p has been found in association with Kap60p and Kap95p (Dilworth et al., 2001) showing that it is capable of interacting with karyopherins. Perhaps an interaction between Nup2p and Kap104p has yet to be identified or possibly another, as yet unknown factor,

remains to be identified that is capable of moving Kap104p import complexes from the NPC to the nuclear interior. This interaction would also provide directionality to Kap104p import.

Further study is also required to determine where the Kap104p/Nab complex dissociates. The knowledge that mRNA and Ran-GTP are required for efficient cargo release (Lee and Aitchison, 1999) suggests the complex dissociates at the site of mRNA transcription. However it is also possible Nab4/Hrp1p and Nab2p are not required until the terminal steps of mRNA processing, allowing them to remain near the NPC instead of being required deep in the nuclear interior. Lee and Aitchison (1999) also showed that RNA alone caused moderate cargo release from Kap104p. It remains to be shown if mRNA causes cargo release from kap104*355-8p or if both mRNA and Ran-GTP are required to release cargo from the mutant.

The recycling of Kap104p also remains unclear. It is presumed that export of karyopherins occurs while they are bound by Ran-GTP. Thus, we expect that Ran-GTP binding would increase the affinity of a karyopherin for export-specific nucleoporins. In an attempt to identify a high affinity binding site for Kap104p/Ran-GTP as it exits the nucleus we used a two-hybrid assay. We showed that Kap104p is unable to interact with Nup42p by two-hybrid analysis, presumably because the presence of Ran-GTP disrupts the interaction. Our results are supported by Allen et al. (2001) who showed that Ran-GTP disrupts the binding of Kap104p to Nup42p. These results suggest Nup42p is not a nucleoporin with which Kap104p interacts

while exiting the nucleus although it does not rule out the possibility that Nup159p or some symmetrical nucleoporin acts as the final binding site for Kap104p recycling.

The use of the mutant kap104*355-8p in the two-hybrid system raises some interesting possibilities. In the two-hybrid system, the interaction between bait and prey occurs in the nucleus, in the presence of Ran-GTP. Testing interactions between Kap104p or kap104*355-8p and nucleoporins may provide some insight into which nucleoporins Kap104p utilizes while entering and exiting the nucleus. Kap104p is predicted to interact only with nucleoporins with which it would associate while bound to Ran-GTP. These are predicted to be capable of interacting with Kap104p while exiting the nucleus. On the other hand, because kap104*355-8p does not efficiently interact with Ran-GTP this mutant is predicted to be capable of interacting with nucleoporins with which Kap104p associates while entering the nucleus. Work done by Allen et al. (2001) has shown Kap104p interacts with Nup116p and Nup100p. They have also shown that these interactions are disrupted by the addition of Ran-GTP (Allen et al., 2001). This raises the possibility that Kap104p docks at Nup42p and moves to interact with Nup100p and Nup116p during import (Figure 17). It is also possible Kap104p interacts with several other nucleoporins during import and these interactions remain to be identified.

The results presented in this thesis provide insight to the role of Ran-GTP in Kap104p mediated import *in vivo*. Our data suggest that Ran-GTP plays a role in promoting Kap104p release from nucleoporins and that this release is necessary for

efficient Kap104p import. The exact Ran-GTP dependent step remains a mystery but further study with the mutant kap104*355-8p may illuminate this site. The results also suggest that Nup42p plays a role in Kap104p docking and Nup116p may be the Ran-GTP dependent step. The potential roles of other nucleoporins in Kap104p import remain to be elucidated. Furthermore, the site of cargo release from Kap104p remains to be determined. Finally, to date all import bound karyopherins are released from all nucleoporins by Ran-GTP. How they then return to the cytoplasm for another round of import remains a mystery. Knowledge of nuclear transport has come a long way but numerous fundamental questions remain unanswered. The tools developed in this thesis will help future work answer some of these questions and provide new insight to the role of Ran in nuclear transport.

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